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Generation and characterization of MMTV-IGF-II transgenic mice revealed that overexpression of IGF-II affects both mammary epithelial apoptosis and proliferation in vivo. IGF-II overexpression inhibited mammary epithelial apoptosis during postlactational involution suggesting that multiple rounds of lactation/involution in the presence of high IGF-II could lead to incomplete involution. In fact, areas of focal epithelial hyperplasia in multiparous transgenic mice have been observed and this aspect is being investigated further. In addition, IGF-II overexpression inhibited mammary epithelial proliferation and this effect appeared to be mediated through IGF-II's ability to increase the levels of the tumor suppressor, PTEN. Thus in normal mammary epithelial cells elevated levels of IGF-II may induce PTEN expression to control the mitogenic and antiapoptotic effects of IGF-II. It is possible that loss of the link between IGF-II and PTEN is one event that occurs prior to IGF-II-induced mammary tumorigenesis. Our preliminary findings support this idea in that we found that PTEN mRNA levels are reduced in MMTV-IGF-II-induced mammary tumors. Finally, IGF-II overexpression driven by the MMTV promoter produced tumors in a number of other organs including the lung and uterus and thus the effects of IGF-II on tumorigenesis in other organs can be explored using this model.

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INTRODUCTION

Among growth factors implicated in breast cancer, insulin-like growth factors (IGFs), especially IGF-II, are gaining increasing recognition as important mitogens. Our previous finding that tissue inhibitor of metalloproteinase (TIMP)-1 modulated IGF-II bioavailability suggested that TIMP-1 may regulate processes other than extracellular matrix degradation during breast cancer progression. To further investigate the role of IGF-II in breast tumorigenesis and its modulation by TIMP-1, we generated transgenic mice in which both IGF-II and Timp-1 have been genetically manipulated. We postulated that overexpression of IGF-II will promote mammary epithelial proliferation eventually resulting in the formation of mammary tumors. In addition the levels of free IGF-II capable of stimulating epithelial proliferation can be regulated by TIMP-1. The overall strategy of this proposal was to generate MMTV-IGF-II transgenic mice that should develop mammary tumors and genetically combine these mice with transgenic mice either overexpressing TIMP-1 (TIMP-1^{high}) or express TIMP-1 antisense RNA (TIMP-1^{low}). Three specific aims were identified; (1) Generate and characterize transgenic mice expressing IGF-II in the mammary tissue, (2) Analyze the course of mammary tumorigenesis, and (3) Establish and characterize double transgenics. We were successful in generating the MMTV-IGF-II transgenic mice however our focus shifted from the original objectives when we found that overexpression of IGF-II did not produce a high incidence of mammary tumors as expected and when we discovered some interesting properties of IGF-II on mammary physiology.

PROPOSAL BODY

Statement of Work, Technical Objective 1: Generate and Characterize Transgenic Mice Overexpressing IGF-II in Mammary Tissue.

To derive transgenic mice overexpressing IGF-II, an expression construct containing a human IGF-II (hIGF-II) cDNA under the control of the MMTV-LTR (Fig. 1a, appendix I) was microinjected into one-cell zygotes. Founder animals were identified by probing Southern blots of *Pst*I cleaved tail DNA with a hIGF-II DNA probe which detected an 833 bp transgene-specific fragment, as well as a 1391 bp endogenous IGF-II fragment (Fig. 1b, appendix I). Three male founders were identified (MI1, MI12 and MI16) and an independent transgenic line established from each founder mouse.

Next we screened for IGF-II expression in the mammary tissue. Mammary mRNA from 35 day-old wild type and transgenic mice was analyzed by RT-PCR using both transgene-specific and control primer sets. A forward primer located in the transcribed portion of the MMTV promoter and reverse primer in the IGF-II transgene (primers 1 and 3, Fig. 1a, appendix I) produced 600-800 bp fragments that were evident only in the mammary tissue of transgenic mice (Fig. 1c, appendix I). The 296 bp fragment generated from the interleukin-2 control primers was visible in every lane and served as a positive control for individual PCR reactions. In addition, in situ hybridization using a digoxygenin-labeled IGF-II antisense riboprobe demonstrated that mammary epithelial cells in transgenic tissue expressed IGF-II and the level of expression was considerably higher compared to wild type controls (Fig. 1d,e appendix I). Therefore, we successfully generated transgenic mice that overexpress IGF-II in the epithelial cells of mammary tissue. During the characterization of these mice we found that IGF-II overexpression inhibited mammary postlactational involution and mammary ductal morphogenesis during pubertal development.

(i) IGF-II Overexpression Inhibited Mammary Epithelial Apoptosis During Postlactational Involution.

A potential mechanism by which IGF-II can promote mammary tumorigenesis is through inhibiting apoptosis. A large body of in vitro and in vivo evidence demonstrates that IGF-I inhibits apoptosis in several cell types [1-7]. On the other hand, the antiapoptotic properties of IGF-II have been shown in vitro, but remain to be proven in vivo. The intracellular molecules responsible for mediating cell survival downstream of the IGF-IR have also not been characterized in vivo. We asked the question whether and how IGF-II overexpression exerts antiapoptotic effects in vivo.

To address this, the event of postlactation mammary involution was selected as a model for our studies. Extensive mammary epithelial proliferation and differentiation culminates in the generation of lobulo-alveoli for lactation. However, these structures undergo scheduled regression following the loss of suckling stimuli, accumulation of milk, and decrease in lactogenic hormones. The lobulo-alveolar collapse is a result of

massive mammary epithelial apoptosis [8;9]. Therefore, postlactation mammary involution offered an appropriate model to uncover the effects of IGF-II on cellular apoptosis and to elucidate the underlying molecular events in a physiological environment.

As an initial measure of the gross mammary alterations that ensue during involution, the weight of mammary fat pads relative to the body weight of the mouse, were monitored. An initial increase in this value was expected after pup removal due to milk accumulation, and was observed in both wild type and transgenic mice at d1i. As involution proceeded, this ratio declined in a characteristic manner leveling out around d4i, a time period of maximal epithelial apoptosis. We found that the mammary gland-to-body weight ratio was consistently higher from d1i to d4i in the MMTV-IGF-II mice compared to wild type controls and these differences were significant at several of the time points (Fig. 3a, appendix I).

To ascertain whether elevated expression of IGF-II resulted in morphological alterations in the epithelial ductal structures, whole mount analysis was performed. At day 10L, the mammary gland is packed with large, milk-filled lobulo-alveoli. Progressive, scheduled involution led to lobulo-alveolar regression followed by clearing of mammary epithelial cellularity and reconstitution of the adipose tissue. We found that mammary involution was delayed in IGF-II overexpressing mice. Specifically, the bud-like, lobulo-alveolar structures persisted for a longer period before regression in MI1 transgenics and these differences were greatest at d4i (Fig. 3b,c appendix I). We also examined the number of structurally intact lobulo-alveoli remaining on specific days of involution. Lobulo-alveolar collapse was clearly delayed in MI1 transgenic mammary glands at d4i (Fig. 3d-g appendix 2) and a higher number of lobulo-alveoli were present from d3i to d6i in the mammary glands of transgenic mice (Fig. 3h appendix I).

To determine the basis of delayed lobulo-alveolar collapse in the transgenic tissue, we elucidated the apoptotic index of mammary epithelial cells throughout involution. Involution in wild type mice showed that apoptotic cells were first detected at d1i and peaked at d4i while the percentage of total mammary epithelial apoptosis in MMTV-IGF-II mice lagged behind that of the controls. Further, when intact lobulo-alveoli were considered, the number of apoptotic cells was approximately half of that observed in the control tissue, from d3i to d5i (Fig. 4c appendix I). Epithelial apoptosis could also be inhibited by implantation of recombinant IGF-II pellets into involuting mammary glands of wild type mice (Fig. 6 appendix I) providing further evidence that IGF-II protein inhibited local epithelial apoptosis.

We further explored the molecular basis of the antiapoptotic effect of IGF-II. A number of in vitro studies have shown that IGF-I and IGF-II inhibit apoptosis through the activation of Akt/PKB [10-13]. We investigated whether reduced mammary epithelial apoptosis in transgenic tissue was linked to activation of Akt/PKB. Phosphorylated Akt/PKB was detected at 10L in both wild type and transgenic mice and its levels increased approximately 5-fold at d1i (Fig. 5A,B, appendix I). In wild type mice, the levels of pAkt/PKB diminished rapidly by d2i and were undetectable thereafter. It was striking that the levels of phosphorylated Akt/PKB remained elevated until d3i and were observed as late as d4i in the MMTV-IGF-II mice. Akt/PKB phosphorylation is regulated by the phosphorylation status of the lipid phosphatidylinositol (3,4,5)-triphosphate (PIP-3). Since the phosphatase PTEN can dephosphorylate PIP-3, levels of PTEN limit Akt/PKB phosphorylation [14-16]. There were no differences in the levels of PTEN protein in the wild type versus the transgenic mammary tissue (Fig. 5c appendix I).

(ii) IGF-II Inhibited Pubertal Mammary Ductal Morphogenesis by Regulating PTEN Expression.

Mammary development begins during embryogenesis and continues postnatally until around 10-12 weeks of age. In response to ovarian and pituitary hormones, terminal end buds (TEBs) form at the tips of the epithelial ducts and ductal lengthening ensues [17]. At day 15 of age a rudimentary ductal tree exists and these epithelial ducts extend into the empty mammary fat pad reaching the lymph node around 35 days of age. Lengthening of the ducts continues until the end of the mammary gland has been reached at around 75 days of age in FVB mice. When the ducts reach the end of the mammary fat pad the TEBs typically disappear [17]. A number of factors in addition to estrogen have been shown to participate in this developmental process including growth hormone [18-20]. More recent studies have demonstrated that growth hormone does not directly affect mammary morphogenesis rather it stimulates the production of IGF-I.

However, the role of IGF-II in this process is poorly understood. Therefore, we examined the effect of elevated IGF-II on ductal morphogenesis.

Although IGF-II is a potent mitogen for mammary epithelial cells and breast cancer cell lines in culture [21-26], we demonstrate here that IGF-II overexpression retards ductal morphogenesis by inhibiting epithelial proliferation. At the whole mount level we found that the length of the mammary epithelial ducts were significantly shorter in the MMTV-IGF-II transgenic mice compared to wild type controls (Fig. 1a-c, appendix II) at day 55 of development. This decrease in ductal length was also observed when pellets containing recombinant IGF-II were implanted into the mammary glands of wild type mice. In addition, there was also a significant reduction in the number of epithelial ducts in the MMTV-IGF-II mice.

To investigate whether these differences in epithelial branching arose during a particular period of mammary development, we also examined mice at days 15, 35 and 75. At days 15 and 35, no significant differences were observed in the length of the epithelial ducts. At day 75 we observed that the MMTV-IGF-II mice have significantly more TEBs remaining in the fourth inguinal mammary gland compared to wild type controls (Fig. 1d,e appendix II). Since TEBs generally disappear once ductal lengthening has finished, these results suggest that ductal lengthening is nearly complete in the wild type mice but still occurring in a substantial number of ducts in the MMTV-IGF-II transgenic mice.

To identify the signaling pathways responsible for the anti-proliferative properties of IGF-II, several signaling molecules downstream of the type I IGF receptor (IGF-IR) were examined. Initially we focused on the PKB/Akt pathway since PKB/Akt activation was recently reported to regulate cell cycle progression [27-29]. A significant reduction in the levels of phosphorylated PKB/Akt was found in the transgenic mammary tissue (Fig. 2a,b appendix II). This finding was intriguing considering the binding of IGF-II to the type I IGF receptor (IGF-IR) normally leads to phosphorylation of PKB/Akt. We reasoned that in the transgenic mammary tissue, either activation of IGF-IR by IGF-II was abrogated or the activity of a molecule responsible for modulating PKB/Akt phosphorylation, such as PTEN, was altered. IRS-1 phosphorylation was used as an indicator of IGF-IR activity because it is phosphorylated by the IGF-IR, and it in turn leads to PI3-kinase and PKB/Akt phosphorylation [30;31]. IRS-1 phosphorylation levels were elevated in the transgenic tissue providing evidence for IGF-IR activation (Fig. 2a appendix II). When we examined PTEN we found that the levels of PTEN protein were significantly elevated in the transgenic tissue (Fig. 2a,c appendix II). Specifically, PTEN was 1.8-fold higher on average in the two transgenic lines compared to wild type tissue. Moreover, the levels of cyclin D1 (Fig. 2a appendix II) were significantly reduced in the transgenic mammary tissue suggesting that the diminished levels of phosphorylated PKB/Akt in this tissue permitted more cylcin D1 degradation. In addition, immunohistochemical analyses using antibodies specific for phosphorylated PKB/Akt and PTEN proteins identified that transgenic mammary epithelium had reduced phosphorylated PKB/Akt and elevated PTEN protein level (Fig. 2d-g appendix II).

We tested whether other signaling pathways implicated in cell proliferation, namely Erk1/Erk2, p38 MAPK, JNK/SAPK, played a role in mediating the effects of IGF-II on mammary epithelial proliferation. There were no significant differences in the levels of phosphorylated Erk1/Erk2 or p38 MAPK in transgenic versus wild type mammary tissue at day 55 (Fig. 2h appendix II). As we were unable to detect phosphorylated JNK/SAPK in the mammary tissue, we assessed ATF-2, which is downstream of JNK/SAPK. The phosphorylation status of ATF-2 was also unaltered in the transgenic mammary tissue (Fig. 2h appendix II). Together, our data show that chronic elevation of mammary IGF-II in the MMTV-IGF-II transgenics induces PTEN protein, restricts PKB/Akt activity, reduces cylcin D1 levels and subsequently inhibits epithelial proliferation and ductal development.

As an alternative means to elevate IGF-II levels, mini-osmotic pumps containing vehicle or recombinant IGF-II were implanted into wild type mice. These pumps release a constant amount of IGF-II over a 14-day period. When PKB/Akt and PTEN were examined we found that the levels of phosphorylated PKB/Akt were significantly reduced while the levels of PTEN were significantly elevated in the mammary tissue of mice receiving a pump containing recombinant IGF-II compared to those mice receiving a pump containing only vehicle (Fig. 3a appendix II). These results confirm that sustained elevation of IGF-II leads to decreased phosphorylated PKB/Akt and increased PTEN protein levels.

To determine whether PTEN gene responds directly to IGF-II, recombinant IGF-II was injected into wild type mouse mammary glands and PTEN mRNA levels assessed. Dramatic increases in the levels of phosphorylated PKB/Akt occurred within 30-60 minutes of IGF-II treatment, confirming that injected IGF-II elicited an immediate cellular response in vivo (Fig. 3b appendix II). The IGF-II administration increased PTEN mRNA levels within 4 hours, with a highly significant 18-fold induction at 8 hours (Fig. 3c appendix II). To date, two studies have reported molecules that regulate the PTEN gene. TGF-β downregulates PTEN expression in vitro [32], and progesterone levels positively correlate with PTEN levels in endometrial tissue [33]. IGF-II is the first molecule identified to alter PTEN expression in vivo.

We also investigated whether the regulation of PTEN was specific to IGF-II. To address specificity, IGF-I or insulin were also injected into wild type mouse mammary glands. Western analysis for the levels of phosphorylated PKB/Akt indicated that IGF-I and insulin induced a similar level of PKB/Akt activation as IGF-II (Fig. 4a appendix II). However, IGF-I and insulin are only about half as effective as IGF-II in elevating the levels of PTEN mRNA (Fig. 4b appendix II). Therefore, IGF-I and insulin can also regulate PTEN expression but they are not as effective as IGF-II. In addition, mammary tissue from transgenic mice that overexpress the wild type ErbB2 receptor (MMTV-neu) was examined. The day-55 MMTV-neu mammary tissue had elevated levels of phosphorylated PKB (2.2-fold) and reduced levels of PTEN (2.5-fold) compared to wild type controls (Fig. 4c appendix II) indicating that increased ErbB2 signaling did not lead to elevated PTEN levels. Thus, regulation of PTEN levels in the mammary gland is restricted to specific receptor tyrosine kinases.

Statement of Work, Technical Objective 2: Analyze the Course of Mammary Tumorigenesis by Assessing Tumor Onset, Proliferation, Angiogenesis and Apoptosis.

As the project evolved so did this objective. MMTV-IGF-II transgenic mice were monitored for the development of mammary tumors and tumors in other organs. We found that mammary tumors rarely develop in MMTV-IGF-II transgenic mice and only 2 out of 44 transgenic females over 6 months of age developed mammary tumors. The MMTV-IGF-II mice did however develop tumors in a number of other organs. Spontaneous tissue abnormalities were found in MMTV-IGF-II transgenic mice as early as 6 months of age and 79% of the mice over one year of age displayed histological alterations such as hyperplasia or overt tumors. The spectrum and frequency of tissues affected is provided in Fig 1b,c appendix III. Male transgenic mice develop alterations in the reproductive organs, lung, lymphatics, and lacrimal gland while female transgenic mice developed alterations in the reproductive tissues (ovary – 34%, uterus – 18%, and mammary – 5%), lung, and lymphatics.

Specifically, compromised lungs showed one or multiple sub-pleural well-circumscribed nodules that histologically were solid epithelial tumors in nature. These tumors were classified as bronchial adenocarcinomas [34] (Fig 2a,b appendix III). Alterations in the male reproductive glands primarily involved the bulbourethral glands. Histological analyses revealed that the walls were lined by mucinous cuboidal epithelial cells, at times forming intraluminal papillae, and constituted by connective fibrous tissue and striated bulbocavernous and ischiocavernous muscle (Fig 2c appendix III). Overall, these alterations were defined as benign mucinous cystadenomas or well-differentiated non-metastatic mucinous cystadenocarcinomas of the bulbourethral glands [35].

Neoplasms of the female reproductive organs were found in the uterus, ovaries and mammary glands. In eight mice the uteri were enlarged and filled with partially necrotic tumors that focally infiltrated the myometrium and serosa (Fig 2d appendix III). One or both ovaries in a number of mice where enlarged due to almost complete replacement of the ovarian stroma and follicles by luteal bodies (Fig 2e appendix III). According to Sass and Rehm [36] these alterations were classified as multinodular ovarian luteomas. One mouse developed a mammary tumor characterized by a unilateral mass that histologically invaded and destroyed adjacent striated muscle and was classified as a well-differentiated type A adenocarcinoma [37] (Fig 2f appendix III).

Some mice developed unilateral overgrowth of the Harderian glands. Tumors were cytologically benign with cuboidal or cylindrical cells displaying foamy, and at times vacuolated and basophilic, mucinous cytoplasm. These spontaneous tumors were classified as acinar adenomas [38].

Finally, several mice developed lymphomas that infiltrated lymphoid and other organs including lungs and kidneys. Histologically and immunohistochemically these tumors were diffuse large cell non-Hodgkin's T-cell lymphomas (Figure 2g-i appendix III). Since the tumor cells stained with an anti-CD3 antibody but not anti-B220 antibody (Fig 2h,i appendix III) they were lymphoblastic in origin [39].

Organ-specific IGF-II expression was investigated to examine whether the ability of IGF-II to induce histological alterations correlated with expression level (Fig 3a appendix III). Analysis non-tumor bearing female transgenic mice showed that transgenic IGF-II levels were highest in the salivary gland, a tissue that did not display any abnormalities. The mammary gland and lung had similar levels of IGF-II expression and these levels were at least 20-fold lower than the salivary gland (Fig 3b appendix III). The mammary tissue rarely developed tumors while tumors were frequently observed in the lungs. Thus, the induction of histological changes by IGF-II does not correlate with expression.

To determine whether the spontaneous tumors in the MMTV-IGF-II mice maintained IGF-II expression, in situ hybridization was performed on a subset of tumors. We found that IGF-II RNA was detectable in tumors arising in the uterus, lung, and mammary (Fig 3g-i appendix III). Since IGF-II was still expressed in the tumors, signaling molecules downstream of the IGF-IR were examined to assess the pathways utilized by these tumors. We focused on the lung tumors since this site was affected in both male and female mice and adequate tissue could be collected for protein analyses. Initially we examined the PKB/Akt pathway since this molecule has been associated with tumorigenesis [40-42]. We found that the levels of phosphorylated PKB/Akt were not elevated in our lung tumor tissue suggesting that induction of IGF-II-induced lung tumors was not dependent on PKB/Akt (Fig 4a appendix III).

Next we looked at Erk1/Erk2 and we found that two of the lung tumors (T2 and T3) had elevated levels of phosphorylated Erk1/Erk2 while the other three did not (Fig 4b appendix III). To further explore signaling downstream of the IGF-IR we investigated the levels of phosphorylated p38 MAPK. We found that the three tumors (T1, T4 and T5) that did not have elevated levels of phosphorylated Erk1/Erk2 had elevated levels of phosphorylated p38 MAPK (Fig 4c appendix III). Since some of the transcription factors activated by Erk1/Erk2 and p38 MAPK overlap we examined two targets that are common to these upstream signaling proteins, Elk-1 and CREB to assess whether these pathways converged on common transcription factors. We were unable to detect phosphorylated Elk-1 in any of the samples however the levels of phosphorylated CREB were elevated in all 5 of the lung tumor samples compared to wild type tissue (Fig 4d appendix III). These results suggested that although different upstream signaling pathways were utilized in individual tumors, these pathways converged on the transcription factor, CREB.

In summary this study showed that overexpression of IGF-II was sufficient to induce sporadic tumors in a number of tissues and that sensitivity to IGF-II-induced tumorigenesis was not strictly dependent on IGF-II expression levels. Further, within the same organ, the lung, IGF-II-mediated tumorigenesis was associated with activation of different upstream signaling molecules. We found that activation of both the Erk1/Erk2 pathway and the p38 MAPK pathway in the IGF-II-induced lung tumors resulted in activation of the transcription factor CREB. This finding may have important therapeutic implications in that treatment modalities targeting upstream signaling molecules may be effective against a subset of tumors while targeting downstream molecules may increase the chances of affecting a greater percentage of the tumors

Statement of Work, Technical Objective 3: Establish and Characterize Double Transgenics and Assess the Ability of TIMP-1 Modulation to Effect the Development and Progression of IGF-II-Induced Mammary Tumors.

This objective was not accomplished for two reasons. First MMTV-IGF-II transgenic mice did not develop mammary tumors with the expected incidence. We have found that only approximately 5% of the MMTV-IGF-II transgenic mice develop mammary tumors (appendix III). With this low tumor incidence we could not address whether MMTV-IGF-II/MMTV-TIMP-1 double transgenic mice had reduced mammary tumor incidence. The second reason that this objective was not accomplished was that our focus changed when we found that IGF-II regulated the tumor suppressor gene, PTEN. Since the discovery of a physiologic link between IGF-II and PTEN had important implications in mammary tumorigenesis we decided to focus our efforts on this research aspect.

Key Research Accomplishments

- Generated MMTV-IGF-II transgenic mice and identified 3 founder animals
- Created PCR screening strategy to genotype subsequent offspring
- Confirmed IGF-II transgene expression in mammary tissue by RT-PCR and In situ hybridization
- Demonstrated that IGF-II expression was elevated 50-100-fold in transgenic mammary tissue compared to wild type mammary tissue.
- Observed a delay in postlactation mammary involution in MMTV-IGF-II transgenic mice characterized by a maintenance of mammary weight and lobulo-alveoli
- Demonstrated a decrease in epithelial apoptosis during mammary involution and showed that this delay was mediated through sustained phosphorylation of Akt/PKB
- Found areas of focal epithelial hyperplasia in multiparous transgenic females
- Observed a delay in mammary ductal morphogenesis in MMTV-IGF-II transgenic mice as characterized by a significant decrease in the number and length of the epithelial ducts
- Demonstrated that the decrease in ductal morphogenesis was mediated by a significant reduction in mammary epithelial proliferation.
- Showed that a decrease in the levels of phosphorylated Akt/PKB with a subsequent decrease in cyclin D1 protein levels could account for the diminished epithelial proliferation
- Showed that reduced phosphorylated Akt/PKB in the MMTV-IGF-II tissue was a result of increased PTEN protein levels
- Found that IGF-II overexpression did not affect mammary physiology during pregnancy.
- Discovered a novel physiologic link between IGF-II and PTEN
- Found that MMTV-IGF-II transgenic mice develop tumors in an number of organs
- Determined that tumor distribution was not related to IGF-II expression level
- Observed that IGF-II-induced lung tumors utilized the Erk1/Erk2 and p38 MAPK but not the PKB/Akt signaling pathways
- Showed that Erk1/Erk2 and p38 MAPK signaling in IGF-II induced lung tumors converged on the transcription factor CREB.

Reportable Outcomes

Employment

Received a faculty position as an Assistant Professor in the Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.

Manuscripts

Moorehead, R.A., Sanchez-Sweatman, O.H., and Khokha, R. Spontaneous IGF-II-Induced Lung Tumors Contain High Levels of Phosphorylated CREB (manuscript in preparation, appendix III)

Moorehead, R.A., Fata, J.E., Hojilla, C., and Khokha, R. IGF-II Regulates PTEN Expression in the Mammary Gland. (manuscript in preparation, appendix II)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Inhibition of mammary epithelial apoptosis through sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice. Cell Death and Differentiation 8:16-29, 2001 (appendix I)

Presentations

Moorehead, R.A., Fata, J.E., and Khokha, R. IGF-II Regulates PTEN Expression in the Mammary Gland. Society of Reproductive Biology, 2001. (presentation)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Inhibition of Mammary Epithelial Apoptosis Through Sustained Phosphorylation of Akt/PKB in MMTV-IGF-II Transgenic Mice. Society of Reproductive Biology, 2000. (presentation)

Abstracts

Moorehead, R.A., Fata, J.E., and Khokha, R. IGF-II Regulates PTEN Expression in the Mammary Gland. Signaling in Normal and Tumor Cells 2001 (abstract)

Moorehead, R.A., Fata, J.E., and Khokha, R. IGF-II Regulates PTEN Expression in the Mammary Gland. Reasons for Hope 2001 (abstract)

Moorehead, R.A., Fata, J.E., and Khokha, R. IGF-II Regulates PTEN Expression in the Mammary Gland. Mammary Gland Biology 2001 (abstract)

Moorehead, R.A., Fata, J.E., Johnson, M.B., and Khokha, R. Delay of mammary involution and epithelial apoptosis in vivo by IGF-II. 5th International Symposium on Insulin Like Growth Factors, 1999. (abstract).

Animal Models

Generated MMTV-IGF-II transgenic mice

Funding Applied For

Department of Defense Breast Cancer Research Program, Idea Award, June 2001
Department of Defense Breast Cancer Research Program, CDA, June 2001

Collaborations

We are establishing a collaboration with Dr. David Flint who wants to breed our MMTV-IGF-II transgenic mice with his IGFBP-5 transgenic mice.

CONCLUSIONS

Generation and characterization of MMTV-IGF-II transgenic mice has revealed that overexpression of IGF-II affects both mammary epithelial apoptosis and proliferation in vivo. Both of these finding have important implications for breast cancer. First, IGF-II overexpression inhibited mammary epithelial apoptosis during postlactational involution suggesting that multiple rounds of lactation/involution in the presence of high IGF-II could lead to incomplete involution. In fact we found evidence that multiple lactation/involution events in our transgenic mice led to areas of focal epithelial hyperplasia and this aspect is being investigated further. Therefore, inhibition of epithelial apoptosis may represent one mechanism through which IGF-II induces mammary tumors. Our other exciting finding was that IGF-II regulates expression of the tumor suppressor gene, PTEN. It appears that in normal mammary epithelial cells, elevated levels of IGF-II induced PTEN expression to control the mitogenic and antiapoptotic effects of IGF-II. It is possible that loss of the link between IGF-II and PTEN is one event that occurs prior to IGF-II-induced mammary tumorigenesis. Our preliminary findings support this idea. We found that PTEN mRNA levels are reduced in MMTV-IGF-II-induced mammary tumors but the limited number of mammary tumors has prevented further investigations. Finally, we did not produce the anticipated number of mammary tumors in the MMTV-IGF-II transgenic mice and this prevented us from examining whether TIMP-1 modulation could affect IGF-II-induced mammary tumorigenesis. Somewhat surprisingly, the MMTV-IGF-II transgenics did develop tumors in a number of other organs and thus the effects of IGF-II on tumorigenesis in other organs can be explored using this model. Thus, although we were unable to accomplish all of our proposed objectives, generation of the MMTV-IGF-II transgenic mice has (i) created a valuable tumor model, (ii) greatly enhanced our understanding of how IGF-II affects mammary epithelial physiology in vivo, and (iii) provided potential mechanisms through which IGF-II overexpression can induce breast cancer.

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Inhibition of mammary epithelial apoptosis and sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice

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Abstract

IGF-II is a growth factor implicated in human cancers and animal tumor models. While the mitogenic properties of IGF-II are well documented, its ability to suppress apoptosis *in vivo* has never been proven. We generated independent MMTV-IGF-II transgenic mice to examine the control of epithelial apoptosis at the morphological, cellular and molecular levels during the physiological event of postlactation mammary involution. Transgenic IGF-II expression was achieved in mammary epithelium and increased IGF-II bioactivity was confirmed by phosphorylation of the insulin receptor substrate-1, a signaling molecule downstream of the type I IGF receptor. IGF-II overexpression induced a delay in mammary involution, as evident by increased mammary gland to body weight ratios and persistence of both functionally intact lobulo-alveoli and mammary epithelial cellularity. The delayed mammary involution resulted from a significant reduction in mammary epithelial apoptosis, and not from increased epithelial proliferation. Recombinant IGF-II pellets implanted into involuting mammary glands of wild-type mice provided further evidence that IGF-II protein inhibited local epithelial apoptosis. At the molecular level, phosphorylated Akt/PKB, but not Erk1 or Erk2, persisted in IGF-II overexpressors and temporally correlated with reduced epithelial apoptosis. Levels of the phosphatase PTEN were unaltered in the transgenic tissue suggesting that the maintenance of Akt/PKB phosphorylation resulted from sustained phosphorylation rather than altered dephosphorylation of PIP-3. Together, this data reveal that IGF-II inhibits apoptosis *in vivo* and this effect correlates with prolonged phosphorylation of Akt/PKB *Cell Death and Differentiation* (2001) **8**, 16–29.

Keywords: IGF-II; transgenic mice; mammary involution; apoptosis; Akt/PKB

Abbreviations: Akt/PKB, protein kinase B; Erk, extracellular signal-regulated kinase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IL-2, interleukin-2; IRS-1, insulin receptor substrate-1; MMTV, mouse mammary tumour virus; PIP-3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homology deleted on chromosome 10

Introduction

By virtue of their ability to affect cell proliferation as well as cell death, survival factors can play a fundamental role in cancer development. Insulin-like growth factors (IGFs) are considered to be typical examples of survival factors. Components of the IGF axis that include the two ligands (IGF-I and IGF-II), the two receptors (IGF-IR and IGF-IIR), and the IGF binding proteins (IGFBPs) are often dysregulated in several human malignancies including breast cancer.[1–3] While the properties of IGF-I that promote tumorigenesis have been extensively studied, the function of IGF-II is not well understood.

IGF-I and IGF-II are secreted molecules with distinct characteristics. Although, they elicit their mitogenic and antiapoptotic actions through the same IGF-IR receptor (reviewed in[4]), the two genes differ *in vivo* in their regulation and function. The levels of IGF-I mRNA increase 10–100-fold in most tissues after birth.[5] In contrast, IGF-II mRNA levels are high during embryogenesis but decline during adulthood.[6] Circulating levels of growth hormone regulate the hepatic and extrahepatic production of IGF-I, while IGF-II production is not intimately linked to growth hormone levels.[7] The significance of IGF-I and IGF-II becomes apparent in animals that lack these genes. Targeted disruption of the IGF-I gene induces death, infertility, and deficiencies in ossification, muscle and lung development, whereas the lack of IGF-II results in viable, fertile, proportionate dwarfs.[8–10] Differences between IGF-I and IGF-II also extend to their role in breast cancer. IGF-II mRNA and protein is more frequently detected in primary tumors and human breast cancer cell lines than IGF-I.[11] In addition, clinical studies show that stromal cells surrounding normal breast epithelium secrete IGF-I while those surrounding the malignant epithelium secrete IGF-II, suggesting that transformation of breast epithelial cells may be associated with a switch from stromal IGF-I to IGF-II expression.[12,13] In rodents, the IGF-II gene is normally imprinted, but is frequently reactivated upon oncogenic transformation in transgenic mice.[14,15] Further, the transgenic expression of IGF-II in mouse mammary tissue leads to the development of mammary tumors after long latency,[16] indicating a causal link between IGF-II activity and mammary tumorigenesis. It is clear that IGF-II plays a role in modulating breast cancer, however the mechanisms underlying its effects remain to be resolved.

A potential mechanism by which IGF-II can promote mammary tumorigenesis is through inhibiting apoptosis. A large body of *in vitro* and *in vivo* evidence demonstrates that IGF-I inhibits apoptosis in several cell types.[17–23] On the other hand, the antiapoptotic properties of IGF-II have been shown *in vitro*, but remain to be proven *in vivo*. The intracellular molecules responsible for mediating cell survival downstream of the IGF-IR have also not been characterized *in vivo*. We asked the question whether and how IGF-II overexpression exerts antiapoptotic effects *in vivo*. To address this, the event of postlactation mammary involution was selected as a model for our studies. Extensive mammary epithelial proliferation and differentiation culminates in the generation of lobulo-alveoli for lactation. However, these structures undergo scheduled regression following the loss of suckling stimuli, accumulation of milk, and decrease in lactogenic hormones. The lobulo-alveolar collapse is a result of massive mammary epithelial apoptosis.[24,25] These events can be synchronized by removal of the litter at a specific day of lactation and have been widely studied at the morphological, cellular and molecular levels.[24,26–31] Therefore, postlactation mammary involution offered an appropriate model to uncover the effects of IGF-II on cellular apoptosis and to elucidate the underlying molecular events in a physiological environment.

We generated and characterized three distinct lines of transgenic mice overexpressing IGF-II in mammary epithelial cells by utilizing the MMTV promoter. Increased IGF-II bioactivity was demonstrated through elevated insulin receptor substrate-1 (IRS-1) phosphorylation. We show, genetically and biochemically, that IGF-II reduced mammary epithelial apoptosis and delayed postlactation mammary involution. Molecular analyses of transgenic mammary tissue revealed that the antiapoptotic effects might be mediated through the sustained phosphorylation of Akt/PKB, an antiapoptotic cytoplasmic protein.

Results

Transgenic IGF-II expression in mammary epithelium of MMTV-IGF-II mice

To derive transgenic mice expressing elevated levels of IGF-II, an expression construct containing the full-length human IGF-II (hIGF-II) cDNA under the control of the MMTV-LTR (Figure 1A) was microinjected into one-cell zygotes. Founder animals were identified by probing Southern blots of *Pst*I cleaved tail DNA with a hIGF-II DNA probe which detected an 833 bp transgene-specific (t-IGF-II) fragment, as well as a 1391 bp endogenous IGF-II (e-IGF-II) fragment (Figure 1B). Three male founders were identified (MI1, MI12 and MI16) and an independent transgenic line established from each founder mouse.

Next we screened for IGF-II expression in the mammary tissue. Mammary mRNA from 35-day-old wild-type and transgenic mice was analyzed by RT–PCR using both transgene-specific and control primer sets. A forward primer located in the transcribed portion of the MMTV promoter and reverse primer in the IGF-II transgene (primers 1 and 3, Figure 1A) produced 600–800 bp fragments (t) that were evident only in the mammary tissue of transgenic mice (Figure 1C). The 296 bp fragment generated from the interleukin-2 control (c) primers was visible in every lane and served as a positive control for individual PCR reactions. To further verify that the 600–800 bp fragments were indeed the result of transgene expression, PCR fragments were transferred to nylon membrane and probed for the presence of the Ha-*ras* sequence that is present in the 5′ untranslated region of the expression construct. As shown in Figure 1C, Ha-*ras* expression (t*) was detected only in transgenic mammary tissue.

To assess whether transgenic IGF-II expression was restricted to the mammary tissue, RT–PCR analysis was performed on a number of organs. We found that salivary gland, spleen, and uterus also expressed t-IGF-II in all three independent transgenic lines while kidney, ovary, liver and pancreas, expressed t-IGF-II in some of the lines (data not shown). These organ-specific expression profiles were consistent with the literature describing MMTV-driven transgenes.[32,33] To compare the level of t-IGF-II expression among the three transgenic lines, quantitative RT–PCR was performed utilizing primer sets for the IGF-II transgene (primers 2 and 3; Figure 1A) and endogenous HPRT. The latter served as an internal control for PCR amplification. These analyses indicated that both MI1 and MI12 mice had considerably higher transgene expression than MI16 mice (data not shown). Thus, we had established three independent MMTV-IGF-II transgenic lines with different levels of ectopic t-IGF-II expression in the mammary gland.

To identify the cell types responsible for IGF-II production during involution, *in situ* hybridization was performed on mammary tissue isolated from day 3 of involution (d3i) using strand-specific IGF-II or Ha-*ras* riboprobes. IGF-II-specific signal was detected in epithelial cells of wild-type tissue, albeit at very low levels (Figure 1D), which is consistent with the findings of Richert and Wood.[34] The transgenic mammary tissue showed IGF-II-specific signal, which localized to epithelial cells (Figure 1E). This signal was much more intense than the e-IGF-II and was present in a majority of epithelial cells. *In situ* hybridization with a Ha-*ras* riboprobe was used to confirm that the transgene expression was confined to the epithelial cells of transgenic animals (Figure 1F, G). Given that stage- and site-specific expression of IGF-II occurred appropriately for our studies, these mice were subjected to further molecular analyses.

Increased IGF-II bioactivity in MMTV-IGF-II mammary tissue

To gauge the amount of transgenic IGF-II in relation to endogenous IGF-II, the levels of e-IGF-II and t-IGF-II mRNA were analyzed in wild-type and transgenic mice during involution by Northern blot and slot blot analyses. Very low levels of e-IGF-II mRNA were evident in mammary tissue from wild-type mice and these levels were maximal on days 10 of lactation and 1 of involution (Figure 2A). On the other hand, transgenic IGF-II mRNA was observed throughout involution, and its expression was 50–100-fold that of the e-IGF-II

mRNA levels (Figure 2A). Thus the level of t-IGF-II was far greater than the e-IGF-II.

The IGFBPs play an important function in the IGF axis. They serve to transport IGFs and modulate their half-life, as well as regulate IGF:IGF receptor interactions (reviewed in[35,36]). Thus, the levels of IGFBPs in a given tissue can influence IGF-II bioavailability. A 50-fold increase in IGFBP-5 and smaller increases in IGFBP-2 and IGFBP-4 have

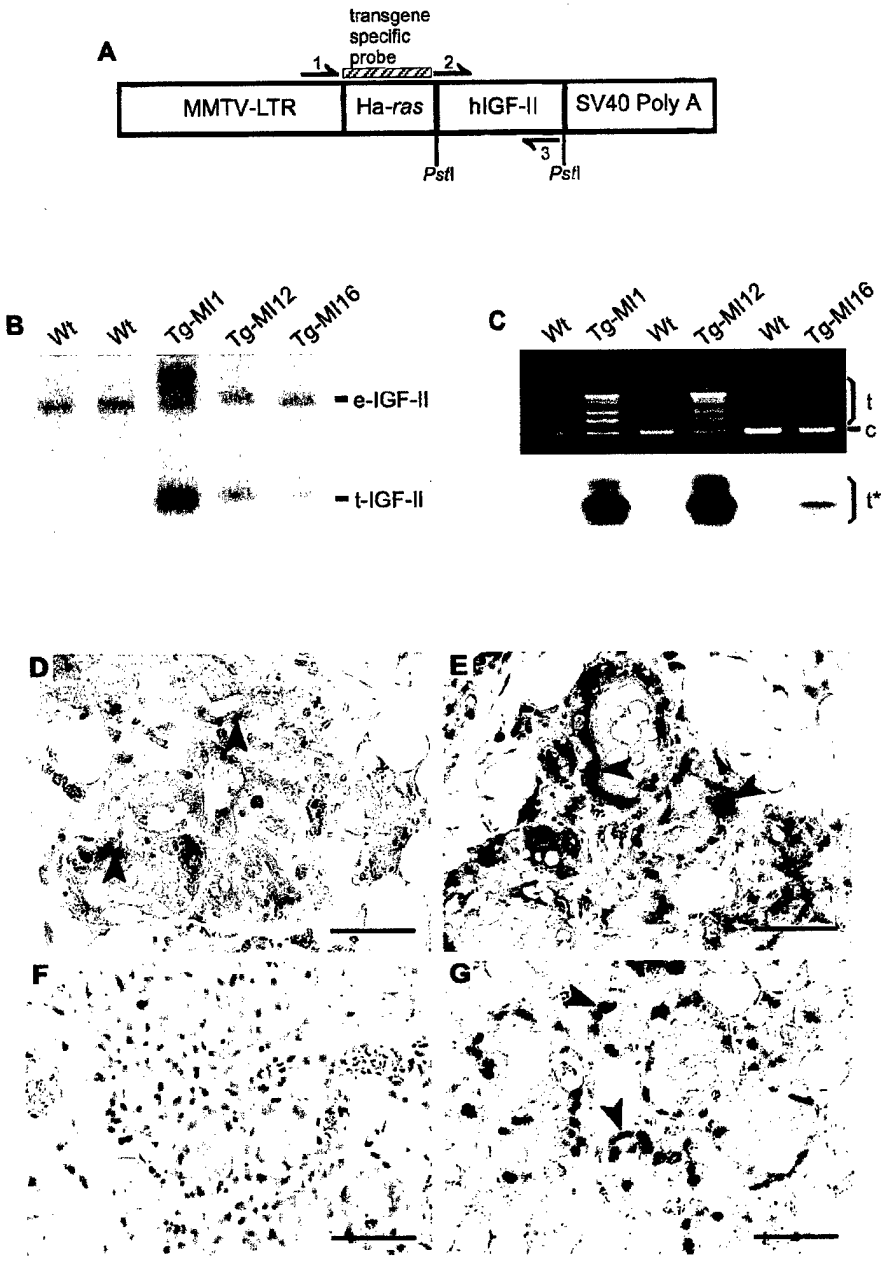


Figure 1 Generation and characterization of MMTV-IGF-II transgenic mice. (**A**) Transgenic expression cassette containing an MMTV-LTR promoter, an untranslated portion of the Ha-*ras* gene, full-length human IGF-II cDNA and a SV40 polyadenylation sequence. Numbers and arrows indicate the location of PCR primers. Primers 1 and 3 were used for RT–PCR while primers 2 and 3 were used for PCR genotyping of MMTV-IGF-II transgenics. (**B**) Southern analysis of IGF-II in wild-type (Wt) mice and MMTV-IGF-II transgenic (Tg) lines MI1, MI12, and MI16. The IGF-II transgene (t-IGF-II) was detectable in all three transgenic lines as an 833 bp fragment while the endogenous IGF-II (e-IGF-II) gene was detected in both wild-type and transgenic mice as a 1391 bp fragment. (**C**) RT–PCR of mammary RNA from 35-day-old MMTV-IGF-II transgenic and wild-type mice using primer sets specific for IGF-II (t; primers 1 and 3) and for a control (c) transcript (IL-2). Transgene expression was restricted to the three transgenic lines while the PCR product for the control transcript was apparent in all samples. Probing with a ^{32}P-labeled Ha-*ras* probe (transgene-specific probe) confirmed that the PCR products were the result of transgene expression. *In situ* hybridization using a Dig-labeled IGF-II riboprobe on sections from (**D**) wild-type or (**E**) transgenic mammary tissue at d3i or a Dig-labeled Ha-*ras* riboprobe in (**F**) wild-type or (**G**) transgenic mammary tissue at d3i. Both probes demonstrated elevated levels of IGF-II expression in the mammary epithelial cells of the transgenic mice compared to controls. Arrowheads indicate positive stained cells; scale bars, 50 μm

been reported in the rat mammary tissue on day 2 of involution.[37] We evaluated whether the levels of IGFBPs were altered in the IGF-II overexpressing mammary tissue, since a concomitant change in these binding proteins would influence the availability of t-IGF-II. Ligand blot analysis revealed that an IGFBP of ~32 kDa (possibly IGFBP-5) peaked at d2i and remained detectable until d4i in both genotypes (Figure 2B). Since the levels were comparable in transgenic and wild-type mice, we concluded that the increased IGF-II production in the transgenic mammary tissue was not confounded by altered IGFBP levels in the tissue. Overall, an increased amount of IGF-II was available to interact with its receptors in the MMTV-IGF-II mammary tissue.

IGF-II induces signal transduction primarily through the IGF-IR,[38] although it has also been reported to stimulate insulin receptor signaling.[39,40] The IRS-1 protein binds to activated IGF-IR, as well as to activated insulin receptor, becomes phosphorylated and propagates signal transduction from both of these receptors. We used immunoblotting with specific antibodies to monitor the levels of phosphorylated IRS-1 (p-IRS-1) and total IRS-1 protein. The ratio of p-IRS-1 to IRS-1 was taken as an indicator of t-IGF-II bioactivity in the transgenic tissue. The levels of p-IRS-1 were considerably higher in IGF-II overexpressing mammary tissue obtained at 10 L, compared to that from controls (Figure 2C). Specifically, the ratio of p-IRS-1 to total IRS-1 in the transgenic tissue was 2.5-fold that of the wild-type tissue (Figure 2D). An increase in the level of p-IRS-1 was also evident in 75-day-old virgin transgenic mammary tissue (data not shown). Together, these observations show that mammary overexpression of IGF-II resulted in increased IGF-II bioactivity in the MMTV-IGF-II mice.

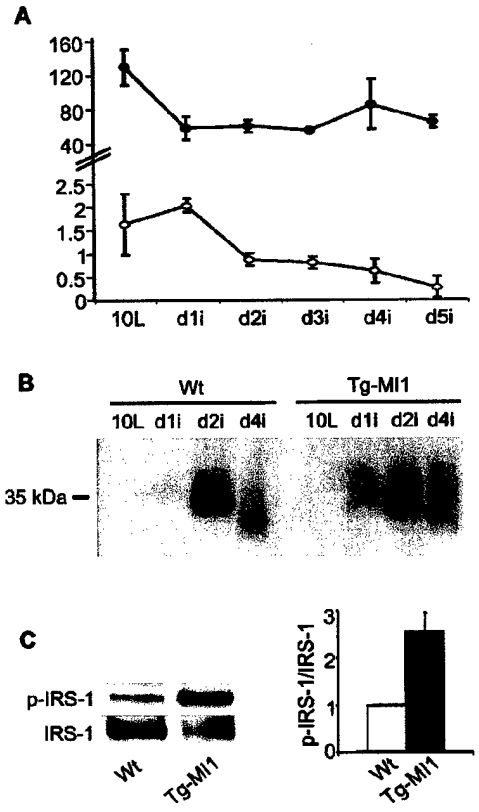


Figure 2 Mammary IGF-II bioactivity in MMTV-IGF-II transgenics. (**A**) Slot blot analysis of e-IGF-II and t-IGF-II mRNA relative to 18 S rRNA in involuting (○) wild-type and (●) transgenic mammary tissue from days 10 L to d5i. (**B**) Ligand blot of IGFBP levels in wild-type and transgenic mammary tissue during involution. (**C**) Western analysis of p-IRS-1 in wild-type and transgenic 10 L mammary tissue. The levels of p-IRS-1 and total IRS-1 were detected using antibodies specific to either the phosphorylated form of IRS or non-phosphorylated IRS-1 and quantified by densitometry

Delayed mammary involution in MMTV-IGF-II transgenic mice

Since extensive epithelial apoptosis is responsible for postlactation mammary involution, we selected this process as a model to delineate the impact of t-IGF-II expression on apoptosis *in vivo*. The following measures were implemented to ensure consistency in this system. We minimized the mouse-to-mouse variation that can arise from the suckling response by maintaining the litter size at five pups per female, and synchronized the onset of mammary involution by removing litters on day 10 L. In addition, all mice were analyzed following their first pregnancy.

As an initial measure of the gross mammary alterations that ensue during involution, the weight of 4th-inguinal mammary fat pads relative to the body weight of the mouse, were monitored. An initial increase in this value was expected after pup removal due to milk accumulation, and was observed in both wild-type and transgenic mice at d1i. As involution proceeds, this ratio declines in a characteristic manner leveling out around d4i, a time period of maximal epithelial apoptosis. We found that the mammary gland-to-body weight ratio was consistently higher from d1i to d4i in the MMTV-IGF-II mice compared to wild-type controls and these differences were significant at several of the time points (Figure 3A).

To ascertain whether elevated expression of IGF-II resulted in morphological alterations in the epithelial ductal structures, whole mount analyses were conducted on each day beginning at 10 L until d8i. At day 10 L, the mammary gland is packed with large, milk-filled lobulo-alveoli. Progressive, scheduled involution then leads to lobulo-alveolar regression that is followed by the clearing of mammary epithelial cellularity and the reconstitution of adipose tissue. We found that the process of mammary involution was delayed in IGF-II overexpressing mice. Specifically, the bud-like, lobulo-alveolar structures persisted for a longer period before regression in MI1 transgenics and these differences were greatest at d4i (Figure 3B vs 3C). It was clearly evident that many lobulo-alveoli were still dilated and contained a secretory substance in the transgenic mice (arrowhead, Figure 3B), whereas these structures had regressed in wild-type mice (Figure 3C). Further, an increase in β-casein mRNA expression relative to 18S rRNA expression was observed

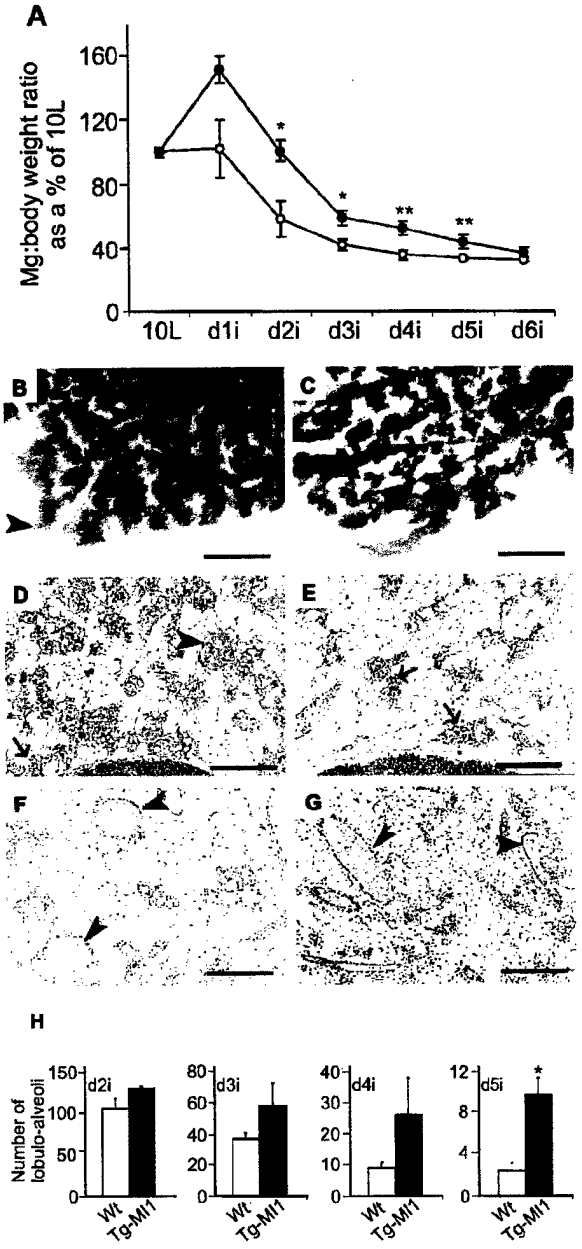


Figure 3 (**A**) Mammary gland (Mg) to body weight ratios during involution in (●) transgenic and (○) wild-type mice. Values are presented as mean ± SEM; (*) $P<0.09$, (**) $P<0.05$. Whole mount analysis of involuting 4th-inguinal mammary glands in (**B**) transgenic and (**C**) wild-type mice at d4i. Arrowheads indicate dilated lobulo-alveoli; scale bars, 800 μm. Hematoxylin/eosin-stained sections of (**D**) transgenic and (**E**) wild-type mice at d4i. Arrowheads indicate structurally intact lobulo-alveoli while arrows indicate collapsed lobulo-alveoli, scale bars, 200 μm. Hematoxylin/eosin-stained sections of d5i involuting mammary tissue in (**F**) MI16 transgenic mice and (**G**) MI1 double hemizygous transgenic mice. Arrowheads indicate structurally intact lobulo-alveoli, scale bars, 200 μm. (**H**) Quantification of the number of structurally intact lobulo-alveoli in wild-type and transgenic mice spanning days 2–5 of involution. Values are presented as mean ± SEM and were considered significantly different (*) when $P<0.05$

in transgenic mammary tissue at 10 L and d1i (data not shown). This suggested that the lobulo-alveoli in the transgenic mice also differed functionally from those in control mice.

Next we performed histological analyses to assess the differences at the cellular level between transgenic and wild-type tissue. The mammary tissue has distinct compartments; connective tissue composed of adipocytes and extracellular matrix and parenchymal tissue composed of epithelial and myoepithelial cells. At 10 L, the mammary gland is composed entirely of a parenchymal compartment with functional lobulo-alveoli and this compartment is removed via apoptosis during mammary involution. Typically, lobulo-alveoli begin to collapse around d3i and cords of mammary epithelial cells predominate by d5i. We performed two measurements: first, we determined the percentage of mammary tissue occupied by the parenchymal compartment and found this value to be increased in involuting transgenic mammary tissue on days spanning d3i-d6i. Significant differences between MI1 transgenic and wild-type mice were observed on d5i and d6i (23.6% ± 1.6 *vs* 11.3% ± 2.1, $P<0.005$ on d5i; and 14.9% ± 1.5 *vs* 6.9% ± 1.2, $P<0.01$ on d6i). Second, we compared the number of structurally intact lobulo-alveoli remaining on specific days of involution. Lobulo-alveolar regression normally occurs earlier in the post-nodal area (from the end of the mammary fat pad to lymph node) than in the pre-nodal area (from lymph node to teat). To maintain consistency we quantified the numbers of lobulo-alveoli in the post-nodal area. Lobulo-alveolar collapse was clearly delayed in MI1 transgenic mammary glands at d4i (Figure 3D *vs* E) and a higher number of lobulo-alveoli were present from d3i to d6i in the mammary glands of transgenic mice than of controls (Figure 3H). This phenotype was not a result of increased epithelial proliferation as the percentage of BrdU positive epithelial cells was not significantly different in the transgenic mammary tissue (data not shown). To rule out the possibility that these phenotypes arose from events other than increased IGF-II expression, such as the site of transgene integration, independent transgenic lines (MI12 and MI16) as well as the MI1 double hemizygous (carrying two IGF-II alleles) mice were subjected to similar analyses on day 5 of involution. An elevation in the mammary cellularity, as indicated by the parenchyma compartment was observed in MI12 (23.6%) and MI16 (26.6%) mice and this value was further increased in MI1 double hemizygous transgenics (37.4%). In addition, multiple intact lobulo-alveoli persisted in MI16 (Figure 3F) and MI1 double hemizygotes (Figure 3G) at d5i, a time when the majority of these structures have regressed in wild-type mice. This demonstrated that the phenomenon of delayed mammary involution occurred consistently in independent MMTV-IGF-II lines.

Decreased mammary epithelial apoptosis in MMTV-IGF-II mice

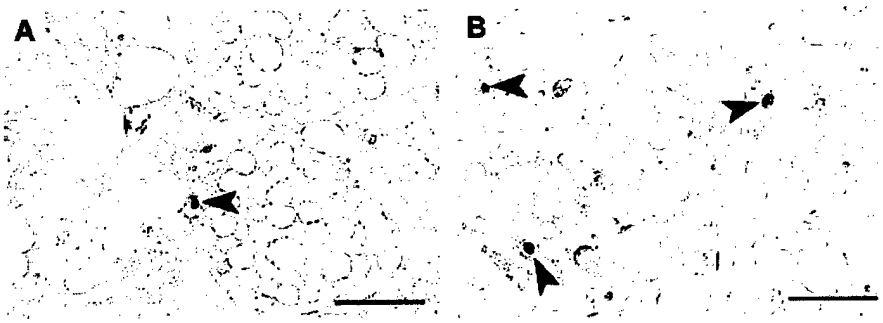
To determine the basis of increased mammary cellularity in the transgenic tissue, we elucidated the apoptotic

index of mammary epithelial cells throughout involution by immunohistochemistry (Figure 4A, B). It is known that programmed cell death is responsible for lobulo-alveolar collapse and removal of epithelial cells. We observed two classes of lobulo-alveolar structures, those that had collapsed and consisted primarily of apoptotic cells, and those appearing essentially intact and consisted primarily of viable epithelial cells. Separate measurements were performed for all apoptotic epithelial cells within the mammary gland and for apoptotic epithelial cells within intact lobulo-alveoli. Kinetics of apoptosis during scheduled involution in wild-type mice showed that apoptotic cells were first detected at d1i and peaked at d4i, which was consistent with published reports.[27,30,41] The percentage of total mammary epithelial apoptosis in MMTV-IGF-II mice lagged behind that of the controls (data not shown). When intact lobulo-alveoli were considered, apoptotic cells could not be detected until d2i (Figure 4C) in the IGF-II overexpressors whereas apoptotic epithelial cells were observed at d1i in wild-type mice. Further, the numbers of apoptotic cells were approximately half of those observed in the control tissue, from d3i to d5i ($P<0.05$, Figure 4C).

Decreased epithelial apoptosis correlates with sustained phosphorylation of Akt/PKB

We further explored the molecular basis of the antiapoptotic effect of IGF-II. A number of *in vitro* studies have shown that IGF-I and IGF-II inhibit apoptosis through the activation of Akt/PKB.[42–45] We investigated whether reduced mammary epithelial apoptosis in transgenic tissue was linked to activation of Akt/PKB. Western blots for Akt and p-Akt spanning 10 L to 4di from two independent sets of animals are presented in Figure 5A. Phosphorylated Akt/PKB was indeed detected at 10 L in both wild-type and transgenic mice and its levels increased approximately five fold at d1i (Figure 5B). In wild-type mice, the levels of p-Akt/PKB diminished rapidly by d2i and were undetectable thereafter. It was striking that the levels of phosphorylated Akt/PKB remained elevated until d3i and were observed as late as d4i in the MMTV-IGF-II mice. Sequential probing of the Western blots provided a measure of total Akt/PKB in the tissue.

Akt/PKB phosphorylation is regulated by the phosphorylation status of the lipid phosphatidylinositol (3,4,5)-triphosphate (PIP-3). Since the phosphatase PTEN can dephosphorylate PIP-3, levels of PTEN can limit the



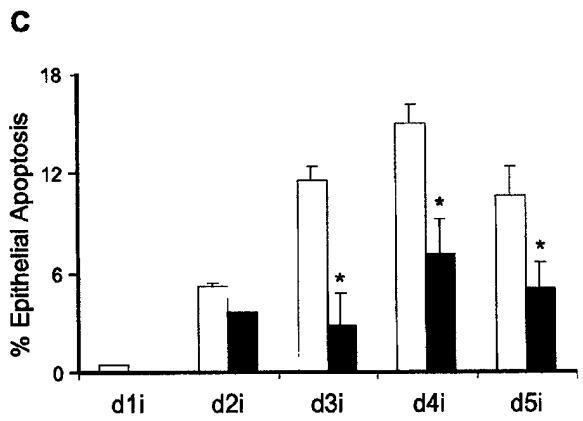


Figure 4 Mammary epithelial apoptosis during involution. Apoptotic epithelial cells were identified immunohistochemically in (**A**) transgenic and (**B**) wild-type d3i mammary tissue. Arrowheads indicate apoptotic cells, scale bars 50 μm (**C**) Percentage of apoptotic epithelial cells comprising structurally intact lobulo-alveoli in (□) wild-type and (■) MI1 transgenic mice. Values represent mean ± SEM of at least three mice at d4i and d5i and at least two mice at all other time points. Differences were considered significant (*) when $P<0.05$

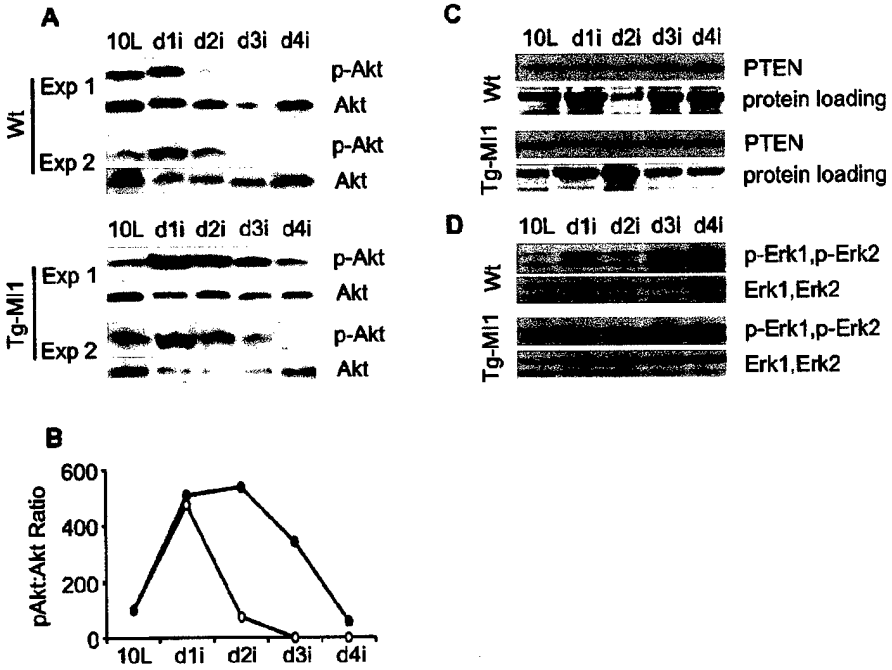


Figure 5 Western analysis of wild-type and transgenic mammary tissue spanning days 10 L to d4i. (**A**) Levels of Akt/PKB as detected by the anti-phospho-Akt(Ser473) and anti-Akt antibodies in two independent sets of animals. (**B**) Quantification of the average levels of phosphorylated Akt/PKB normalized to the total levels of Akt/PKB in the two experiments in (○) wild-type and (●) transgenic mice. Values on the Y-axis are arbitrary units. (**C**) Levels of PTEN protein. Lower panels represent the amido black-stained membrane to show protein loading. (**D**) Levels of phosphorylated Erk1 and Erk2 as well as total Erk 1 and Erk 2 protein

duration of Akt/PKB phosphorylation.[46–48] There were no differences in the levels of PTEN protein in the wild-type *versus* the transgenic mammary tissue (Figure 5C). Interestingly, it appeared that the levels of PTEN protein began to increase at d3i, the time the Akt/PKB phosphorylation was lost.

Although the downstream targets of Akt/PKB are still poorly understood it has been demonstrated that Akt/PKB can phosphorylate Bad in tissue culture systems (reviewed in[49–51]). We attempted to assess the phosphorylation status of Bad in our mammary tissue. The phosphorylation-specific antibody (Ser112) was unable to detect phosphorylated Bad during the events of physiological apoptosis (data not shown).

Activation of Erk1 and Erk2 has also been implicated in inhibiting apoptosis, possibly through phosphorylation of Bad.[52,53] Since IGF-II-mediated signaling through the IGF-IR can stimulate the Erk pathway[4] we examined the levels of phosphorylated Erk1 and Erk2 to seek the involvement of this pathway in the antiapoptotic response of IGF-II. The levels of phosphorylated Erk1 and Erk2 were lowest at 10 L and increased as involution proceeded. There were no differences in Erk1 or Erk2 in the wild-type and transgenic involuting mammary tissue. If these proteins had a function in the physiological antiapoptotic response of IGF-II, higher levels of phosphorylated Erk1/Erk2 at 10 L to d2i would be expected. Together, this data revealed that Akt/PKB is possibly involved in the antiapoptotic signaling of IGF-II *in vivo*.

Normal postlactation epithelial apoptosis is inhibited by IGF-II slow-release pellets

Low levels of IGF-II exist during murine mammary morphogenesis,[34] and we had observed low IGF-II expression during the period of postlactation involution (Figure 2A). To investigate whether elevating IGF-II during normal mammary involution would directly inhibit mammary epithelial apoptosis, a biochemical approach was used with topical application of recombinant IGF-II. Elvax-40 slow-release pellets containing 500 ng of rIGF-II were implanted in the 4th-inguinal mammary gland of wild-type mice on d2i, in the post-nodal area. An inert control pellet that lacked IGF-II was implanted in the contralateral tissue of each mouse. Due to the identical hormonal milieu of the two glands, the contralateral tissue serves as an ideal control. Epithelial apoptosis surrounding either a pellet containing rIGF-II or a control pellet, detected immunohistochemically, is shown in Figure 6A, B respectively. A significant decrease in the apoptotic index of epithelial cells in the immediate vicinity of the implanted rIGF-II pellets was found in tissue removed at d5i (Figure 6C). This provided an independent confirmation that an elevation of IGF-II protein directly inhibited the normal mammary epithelial apoptosis.

Discussion

We have generated independent MMTV-IGF-II transgenic mice and examined epithelial apoptosis at the morphological,

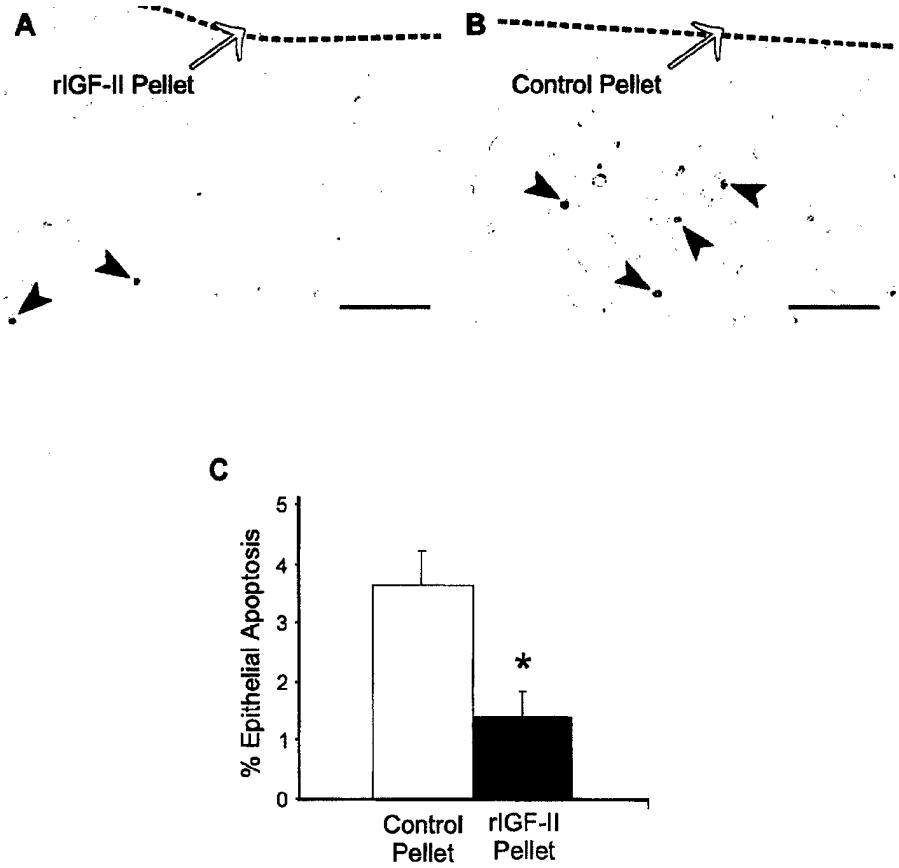


Figure 6 Mammary epithelial apoptosis 3 days after implantation of (**A**) a pellet containing rIGF-II or (**B**) a control pellet in the 4th-inguinal mammary gland of wild-type mice. Arrowheads indicate apoptotic cells, scale bars 50 μm. (**C**) Quantification of the percentage of mammary epithelial cells undergoing apoptosis surrounding either (□) a control pellet or (■) a pellet containing rIGF-II. Values represent mean $\pm$ SEM of five animals and these values were considered statistically significant (*) when $P<0.05$

cellular and molecular levels during the physiological event of postlactation mammary involution. Here we provide the first *in vivo* demonstration of the anti-death role of IGF-II during physiological apoptosis. The antiapoptotic effect of IGF-II was initially evident in the involuting mammary glands as a delayed onset of mammary weight loss, slower lobulo-alveolar involution of mammary tissue at the morphological level, and higher epithelial cellularity at the tissue level, when compared to controls. This resulted from a delayed onset of epithelial apoptosis rather than altered proliferation. The antiapoptotic effect of IGF-II was confirmed biochemically. Elevating IGF-II protein levels through implantation of a slow-release pellet directly led to a local reduction of epithelial apoptosis within the involuting tissue. Downstream of the IGF-IR, sustained phosphorylation of Akt/PKB throughout the period of delayed epithelial apoptosis confirmed that this negative regulator of apoptosis contributed to the antiapoptotic effects of IGF-II *in vivo*. This study provides further evidence that the contribution of IGF-II to breast tumorigenesis may extend beyond its known proliferative capacity.

Role of the IGF axis in mammary involution

The survival of mammary alveolar epithelial cells during lactation is dependent on a number of factors including prolactin and growth hormone. It was originally proposed that growth hormone suppressed alveolar epithelial apoptosis through elevating the levels of IGF-I and/or IGF-II.[54] However, subsequent studies in which rats were subcutaneously administered IGF-I, IGF-II or IGFs complexed with IGFBP-3, failed to mimic the survival effects of growth hormone during involution.[55,56] Such an inability of the IGFs to inhibit apoptosis has been attributed to changes in the levels of IGFBPs that concomitantly occur during involution.[37,57] It is thought that IGFs present in the milk act as survival factors, but the elevation of IGFBPs during involution effectively sequester the IGFs. This prevents the interaction of IGFs with the IGF-IR, essentially removing the IGF survival signal from mammary epithelial cells.[37,57] In support of this concept are the findings that when sufficient levels of IGF-I are attained through transgenic overexpression, IGF-I suppresses apop-

tosis and promotes epithelial cell survival during mammary involution.[17,58]

The involvement of IGF-II in mammary involution has not been studied. Until a recent publication by Richert and Wood[34] it was generally believed that IGF-II was not expressed in adult murine mammary tissue.[8,59] Our *in situ* hybridization with a riboprobe specific for IGF-II extended the findings of Richert and Wood[34] in that IGF-II was also expressed during postlactation involution within mammary epithelial cells, albeit at low levels. Further, we found that the levels of endogenous IGF-II increased from 10 L to d1i and declined thereafter, suggesting that it may play a role in mammary involution. The decrease in endogenous IGF-II expression after d1i, coupled with a dramatic increase in IGFBP levels at d2i, likely removes the IGF-II-mediated survival signals. In our genetic approach, much higher levels of IGF-II were achieved throughout involution in the transgenic tissue. This IGF-II expression, in the absence of concurrent IGFBP elevation, maintained IGF-II-mediated survival signaling and delayed mammary epithelial apoptosis and lobulo-alveolar collapse. The same effect was obtained when a biochemical approach was used. Implantation of IGF-II pellets in the mammary tissue of wild-type mice inhibited local mammary epithelial apoptosis. These observations highlight the potential for IGF-II, like IGF-I, to regulate natural mammary involution.

The antiapoptotic property of IGF-II may underlie its tumorigenic potential

Based on previous studies in transgenic systems, a causal relationship has emerged between IGF-II expression and cancer formation in several tissues (reviewed in[11]). For example, the transgenic expression of IGF-II has been associated with lymphomas and hepatocellular carcinomas[60] (and reviewed in[61]), and its expression from a mammary-specific sheep β-lactoglobulin promoter leads to mammary tumorigenesis.[16] Further, the normally silent IGF-II gene often becomes reactivated during viral oncogene-driven tumorigenesis, such as that observed in pancreas[15] and liver.[14] While IGF-II expression is temporally coordinated with the onset of proliferation in some of these models,[14,15,62] it is conceivable that its antiapoptotic property also plays a crucial role in the genesis of cancer. This may be especially applicable to the mammary gland since this tissue experiences repeated and extensive epithelial turnover throughout female life; during each round of the estrous cycle,[63] (Fata and Khokha, unpublished data) and postlactation involution.[24,26-31]

Our investigations used both genetic and biochemical approaches to conclusively show that IGF-II suppressed mammary epithelial apoptosis *in vivo* during postlactation mammary involution. Based on these observations we propose that the low incidence of mammary tumorigenesis seen in IGF-II transgenics such as the β-lactoglobulin-IGF-II mice,[16] may be initiated by incomplete epithelial apoptosis, a hypothesis that was not examined by this group. It has been shown that reduced mammary epithelial apoptosis due to Bcl-2 overexpression, although insufficient to confer tumorigenesis, enhances the development of MMTV*myc*-induced mammary tumors.[64] These findings of reduced apoptosis are relevant since decreased mammary epithelial apoptosis is associated with an increased risk of fibrocystic changes and the development of carcinoma in premenopausal women.[65] We are currently breeding our MMTV-IGF-II transgenic mice with mice that overexpress the *neu* oncogene to examine whether IGF-II-mediated inhibition of epithelial apoptosis augments *neu*-induced mammary tumorigenesis.

In vivo molecular targets of IGF-II survival signaling

We extended our finding of IGF-II-mediated inhibition of mammary epithelial apoptosis to the molecular level by examining downstream antiapoptotic signaling molecules. *In vitro*, the IGFs have been shown to inhibit apoptosis through the activation of Akt/PKB.[42-44] Our analyses of the total and phosphorylated levels of Akt/PKB revealed that p-Akt/PKB was detectable in MMTV-IGF-II mammary tissue until d4i, while it disappeared after d2i in wild-type mice. The temporal decline of p-Akt/PKB coincided with the peak of epithelial apoptosis during involution in both transgenic and wild-type mice. Specifically, minimal levels of apoptosis were observed at times of high levels of p-Akt/PKB, and high levels of mammary epithelial apoptosis were seen immediately after the levels of p-Akt/PKB had substantially declined. Thus, the dynamics of Akt/PKB phosphorylation were consistent with its role as a negative regulator of apoptosis.

The dephosphorylation of Akt/PKB may, in part, relate to the changing levels of IGFBPs. Consistent with our finding that IGFBP levels peaked at d2i in the mouse, a dramatic increase in IGFBPs has been reported at day 2 of involution in rats.[37] Day 2i is also the time when we observed a rapid decline in the levels of p-Akt/PKB in wild-type mice. An increase in IGFBP levels at d2i likely reduced IGF bioavailability, which in turn, limited the downstream phosphorylation of Akt/PKB. Based on these findings we propose that t-IGF-II bioactivity and Akt/PKB phosphorylation was sustained in the MMTV-IGF-II transgenic tissue until d4i due to the high IGF-II:IGFBP ratio. The fact that IGFBP levels were comparable to those in wild-type mice while IGF-II expression was increased in the MMTV-IGF-II mice likely resulted in an abundance of free IGF-II available to stimulate IGF-IR and activate Akt/PKB.

Another potential regulator of Akt/PKB phosphorylation is the phosphatase PTEN. This molecule dephosphorylates PIP-3, which is then unable to promote Akt/PKB activation.[46-48] We did not observe a differential regulation in the levels of PTEN protein among the wild-type and transgenic mice suggesting that the maintenance of Akt/PKB phosphorylation was primarily driven by the increased availability of IGF-II rather than a reduction in the levels of PTEN. The eventual loss of Akt/PKB phosphorylation in transgenic mice may relate to the dependence of mammary epithelial cell survival on other parameters such as the contact with the extracellular matrix molecules, as discussed in the next section.

Exactly how Akt/PKB inhibits apoptosis remains unresolved. It has been proposed that Akt/PKB phosphorylates the proapoptotic molecule Bad and prevents its interaction with Bcl-2 thus suppressing cytochrome *c* release from

mitochondria and subsequent apoptosis.[51] We were unable to detect phosphorylated Bad in our involuting mammary tissue. This result was not completely unexpected as Bad is expressed at very low levels.[51] In humans, another proposed downstream antiapoptotic target of Akt/PKB is caspase 9. Akt/PKB can phosphorylate human caspase 9 on Ser196 and inhibit its protease activity,[66] however, this phosphorylation site is absent in mice[56] and thus the relevance of caspase 9 in mediating the antiapoptotic effects of Akt/PKB in murine tissue is unclear.

IGF-II also has the potential to inhibit apoptosis through the activation of Erk1 and Erk2.[52,53] Since there was no difference in the levels of phosphorylated Erk1 or Erk2 in our transgenic mammary tissue it appears that this pathway does not significantly contribute to the antiapoptotic effects of IGF-II during involution.

The antiapoptotic effects of IGF-II are influenced by structural factors

Both soluble factors and contact with the basement membrane are critical for epithelial cell survival.[24,26,27,67,68] When mammary epithelial cells are cultured on basement membrane, apoptosis induced by the withdrawal of lactogenic hormones can be inhibited by the addition of IGF-I or IGF-II.[69] Also, IGF-mediated phosphorylation of IRS-1 and its association with PI-3 kinase are enhanced in cells cultured on basement membrane suggesting that antiapoptotic signaling from the IGF-IR is more efficient when mammary epithelial cells are in contact with the basement membrane.[69] Further, the IGFs are incapable of preventing apoptosis in the presence of an antibody that blocks the binding of mammary epithelial cells to laminin.[69] Overall these studies indicate that soluble factors, such as the IGFs, act in concert with structural that blocks the binding of mammary epithelial cells to laminin.[69] Overall these studies indicate that soluble factors, such as the IGFs, act in concert with structural signals, such as the extracellular matrix, in determining epithelial apoptosis. This also likely explains why transgenic IGF-II expression only delayed mammary gland involution and did not completely inhibit this process. The role of basement membrane degradation during involution and the regulation of the cleavage of IGFBPs by the matrix metalloproteinases, and the inhibition of this process by tissue inhibitors of metalloproteinases are currently under investigation in our lab.

Phenotypes in IGF-II overexpressing transgenic mice

Human IGF-II has been used to generate a number of transgenic strains that develop organ abnormalities as well as lymphomas, hepatocellular carcinomas and lung adenocarcinomas.[65,70–78] With respect to IGF-II overexpression in the mammary tissue, Bates *et al*[79] have used the sheep β-lactoglobulin promoter and demonstrated mammary tumor formation albeit at a relatively low tumor incidence. This promoter induces transgene expression primarily during lactation.[80–82] In contrast, our use of the MMTV promoter resulted in transgene expression at all stages of mammary physiology: ductal development, lactation and involution. Our oldest mice are ~18 months and have yet to show overt mammary tumors. However, we have observed focal areas of epithelial hyperplasia in mammary glands of multiparous transgenic mice as well as sporadic tumors in other organs where MMTV-directed IGF-II is expressed (Moorehead and Khokha, unpublished observations). At present, it remains unresolved why IGF-II overexpression driven by the β-lactoglobulin promoter but not the MMTV promoter induces mammary tumors. It is difficult to compare the β-lactoglobulin-IGF-II and MMTV-IGF-II mice since the basal level of transgene expression in the mammary tissue prior to tumor formation and the number of pregnancies required has not been reported for the β-lactoglobulin mice.[16]

In summary, this study represents the first report on the ability of IGF-II to inhibit physiological apoptosis. We also show that this antiapoptotic effect correlates with sustained phosphorylation of Akt/PKB and not Erk1 or Erk2. Based on the observation that PTEN protein levels are unaltered, we propose that the sustained phosphorylation of Akt/PKB in the MMTV-IGF-II transgenics was propagated by continual IGF-II-mediated stimulation of molecules upstream of Akt/PKB rather than their lack of dephosphorylation. These findings represent a potential mechanism through which IGF-II overexpression may predispose the mammary gland to tumor development.

Materials and methods

The MMTV-IGF-II transgenic mice

We made an MMTV-IGF-II expression vector by cloning an 833 bp *Pst*I-*Pst*I full-length human IGF-II cDNA (from Dr. KJ Cullen, Georgetown University) downstream of the MMTV-LTR promoter (from Dr. W Muller, McMaster University). The MMTV-IGF-II construct also included an untranslated region of the Ha-*ras* gene and a SV40 polyadenylation sequence (Figure 1A). DNA was prepared for microinjection by digesting the MMTV-IGF-II expression vector with *Sal*I and *Spe*I. The cleaved DNA was electrophoresed through a 1% agarose gel and the transgenic cassette purified with a GeneClean II Kit (Bio/Can Scientific, Mississauga, ON, Canada) using the manufacturer's protocol. The expression construct was microinjected at a concentration of 1 ng/μl into the pronuclei of zygotes from FVB mice and microinjected zygotes were transferred to pseudopregnant FVB females.

Tail biopsies were taken from potential founders and incubated overnight at 55°C in a digestion buffer containing 20 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS and 0.4 mg/ml Proteinase K. Tissue digests were agitated for 5 min using an Eppendorf mixer and 250 μl of 6 M NaCl was added to each sample followed by an additional 5 min of mixing. Samples were centrifuged at 20 000 × *g* for 7 min and DNA was precipitated with isopropanol. Ten micrograms of tail DNA was incubated overnight with *Pst*I at 37°C and the DNA was separated on a 1% agarose gel. Following transfer to Genescreen Plus nylon membrane (NEN Life Science Products, Boston, MA, USA), blots were probed with a ^{32}P-dCTP-labeled human IGF-II probe. All ^{32}P-labeled probes used in the present study were generated by random priming using a Prime-It II random primer labeling kit (Stratagene, La Jolla, CA, USA) following the manufacturer's protocol. Membranes were pre-hybridized and hybridized in Church buffer (7% SDS, 1% BSA, 0.5 M NaPi pH 7.2, 0.001 M EDTA) at 65°C. Washes were performed as follows, 0.5% SSC/0.1% SDS for 10 min at room temperature, 0.5% SSC/0.1% SDS for 10 min at 65°C, and

twice in 0.2% SSC/0.1% SDS for 10 min each at 65°C. Membranes were developed in phosphoimager cassettes (Molecular Dynamics, Sunnyvale, CA, USA) and visualized using a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA)

For routine genotyping, tissue was incubated overnight at 55°C in 50 μl of digestion buffer. The samples were diluted to 400 μl with water and 1 μl was amplified using a forward primer (5′-CCGAGAGGGACGTGTCGA-3′) and a reverse primer (5′-GCCTCCCTGAACGCCTCG-3′) both located in the IGF-II transgene (primers 2 and 3; Figure 1A). Forward (5′-CTAGGCCACAGAATTGAAAGATCT-3′) and reverse (5′-GTAGGTGGAAATTCTAGCATCATCC-3′) primers for the mouse interleukin-2 gene were also included in each reaction and served as a positive control. The IGF-II and interleukin-2 primers produced 187 bp and 324 bp bands, respectively.

RT–PCR and quantitative RT–PCR

RNA was extracted from mammary tissue using the method of Chomczynski and Sacchi.[83] RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco BRL, Burlington, ON, Canada). The resulting cDNAs were amplified using a forward primer (5′-GCCATCCCGTCTCCGCTCGTCACTTATC-3′) located in the transcribed segment of the MMTV promoter and a reverse primer (5′-GCCTCCCTGAACGCCTCG-3′) located in the IGF-II transgene (primers 1 and 3; Figure 1A). Forward and reverse primers for interleukin-2 (described above) were used to ensure that each PCR reaction worked. PCR products were separated on a 1.8% agarose gel and transferred to nylon membrane. Transgene-specific expression was confirmed using a ^{32}P-dCTP-labeled Ha-*ras* probe (transgene-specific probe, Figure 1A) and hybridization conditions described above.

The level of transgene expression was determined in mammary glands from 35-day-old female transgenics and their littermate controls using quantitative RT–PCR. RNA was reverse transcribed and amplified using a forward and reverse primer specific for IGF-II transgene (described above) and a forward (5′-GTTGGATACAGGCCAGACTTTGTTG-3′) and reverse primer (5′-GATTCAACTTGCGCTCATCTTAGGC-3′) for the house keeping gene hypoxanthine phosphoribosyl transferase (HPRT). It was determined that 23 cycles of PCR fell within the linear range of amplification for both the IGF-II primers and the HPRT primers. Following 23 cycles of PCR, samples were electrophoresed through a 1.8% agarose gel, transferred to nylon membrane and probed with either a ^{32}P-dCTP-labeled human IGF-II or a ^{32}P-dCTP-labeled HPRT probe. Membranes were developed and the radioactive signal from IGF-II was quantified relative to that of HPRT using a phosphoimager as described above.

Slot blot analysis

Two micrograms of mammary RNA was heated to 68°C for 15 min in a solution containing 50% formamide, 7% formaldehyde and 1 x SSC. Two volumes of 20 x SSC was added to each sample and the samples were loaded on to nylon membranes (pre-soaked in 20 x SSC for 1 h) using a Minifold II slot-blot system (Schleicher & Schuell, Keene, NH, USA) followed by gentle suction. Each well was rinsed once with 10 x SSC followed again by gentle suction. Hybridization of the membrane with the appropriate ^{32}P-labeled probe and subsequent washes were performed as described above.

In Situ hybridization

Antisense probes for human IGF-II or Ha-*ras* were generated by incorporating digoxygenin (DIG)-labeled UTP (Boehringer Mannheim, Laval, PQ, Canada) following the manufacturer's protocol. DIG *in situ* hybridization was performed essentially as previously described[84,85] with the following modifications. Specifically, tissue was fixed in 4% (w/v) buffered formalin overnight, tissue sections were treated with 20 μg/ml of Proteinase K for 20 min at room temperature and sections were washed three times in 4 x SSC for 15 min each at room temperature.

Western analysis and ligand blotting

Mammary tissue was homogenized in lysis buffer (10 mM Tris pH 7.6, 5 mM EDTA, 50 mM NaCl, 1% Triton-X, 30 mM tetra-sodium pyrophosphate, 500 μM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin) and lysates were collected following centrifugation at 15 000 x *g* for 20 min at 4°C. Protein concentrations were determined using Bradford assay reagents (Bio-Rad, Hercules, CA, USA). Reduced proteins were separated through 8% polyacrylamide gels for Akt, PTEN, and Erk1/Erk2 and 6% gels for IRS-1 using an Xcell II min cell system (Novex, San Diego, CA, USA). Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 25 V for 90 min and the membranes were blocked in 5% skim milk in tris-buffered saline containing 1% tween (TBST) for 2 h at room temperature. Primary antibody for Akt, phosphorylated Akt(Ser473), Erk1/Erk2 and phosphorylated Erk1/Erk2(Thr202/Tyr204) (New England Biolabs, Beverly, MA, USA) were incubated at a dilution of 1:1000 in 5% skim milk in TBST with the membranes overnight at 4°C. The primary phospho-IRS-1 antibody (Ser612) (Medicorp, Montreal, QU, Canada) was used at a concentration of 0.5 μg/ml and the primary IRS-1 antibody (Upstate Biotechnology, Lake Placid, NY, USA) was used at a 1:1000 dilution. The antibody for PTEN (New England Biolabs, Beverly, MA, USA) was used at a dilution of 1:1000 in 5% BSA in TBST. Proteins were detected using an HRP-conjugated anti-rabbit secondary antibody and LumiGLO reagents (New England Biolabs, Beverly, MA, USA) and were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C. Ligand blotting of wild-type and transgenic involuting mammary tissue was performed as we have previously described.[14]

Whole mount analysis

The 4th-inguinal mammary glands were removed from mice and placed on glass slides. After air-drying for 10 min, the mammary tissue was fixed overnight in Clarke's solution (75% ethanol, 25% glacial acetic acid). Fixed mammary glands were dehydrated in 70% ethanol for 30 min and stained overnight in carmine alum (0.2% carmine (w/v) and 0.5% aluminum potassium sulfate (w/v)). The mammary glands were then destained (35% HCl, 70% ethanol) for 3–4 h, dehydrated in increasing concentrations of ethanol and defatted in toluene. Images were captured using a Sony 3CCD color video camera attached to a Leica MZ12 microscope (Leica Microsystems Inc., Buffalo, NY, USA) and Northern Eclipse Software (Empix Imaging Inc. Mississauga, ON, Canada).

Immunohistochemistry and histological staining

Mammary glands were fixed in 4% (w/v) buffered formalin overnight at room temperature prior to embedding. Paraffin sections were dewaxed in toluene and re-hydrated in decreasing concentrations of alcohol. To detect apoptotic cells in wild-type and transgenic involuting

mammary tissue, sections were digested with Proteinase K (20 μg/ml) at room temperature for 15 min and terminal end-labeling of fragmented DNA in apoptotic cells was performed using an Apop Tag *in situ* apoptosis detection kit (Intergen, Purchase, NY, USA) following the manufacturer's protocol. Detection of apoptotic cells in the pellet experiments and bromodeoxyuridine (BrdU) immunohistochemistry were performed as previously described.[86,87]

Elvax-40 slow release pellets

Elvax-40 pellets containing recombinant human IGF-II (rIGF-II) (Calbiochem, San Diego, CA, USA) were generated as previously described.[87] Pellets containing 500 ng of rIGF-II were implanted in the 4th-inguinal mammary gland of wild-type mice at day 2 of involution. Control pellets containing only the vehicle (PBS) were implanted in the contralateral mammary gland. The mice were sacrificed 3 days later and the 4th-inguinal mammary glands along with the control or IGF-II pellets were isolated for analysis.

Statistics

All values are presented as mean $\pm$ SEM. Statistical significance was determined using the Student's *t*-test and values were considered significant when $P < 0.05$.

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IGF-II Regulates PTEN Expression in the Mammary Gland

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Background: PTEN is at the crux of cell survival due to its ability to regulate the phosphorylation status of the cell survival kinase, PKB/Akt. However, factors that regulate the expression of the tumor suppressor gene PTEN remain elusive. We reasoned that IGFs would impact PTEN expression given their capacity to influence PKB/Akt phosphorylation and cell survival.

Results: We found that IGF-II regulates mammary epithelial PTEN expression in vivo. Transgenic overexpression of IGF-II inhibited mammary ductal development, epithelial proliferation, PKB/Akt phosphorylation, and cyclin D1 levels while increasing PTEN protein levels. Inhibitory effects of IGF-II on ductal development were confirmed by transplanting transgenic epithelial cells into wild type fat pads and through implantation of IGF-II pellets into wild type mammary tissue. Elevated PTEN protein and reduced phosphorylated PKB/Akt levels were also observed in mammary tissue from mice containing recombinant IGF-II osmotic pumps. The ability of IGF-II to regulate PTEN expression was demonstrated through direct IGF-II administration to wild type mammary glands. IGF-I and insulin were also capable of increasing PTEN mRNA levels but the magnitude of PTEN induction was only half that induced by IGF-II. In contrast, transgenic overexpression of ErbB2 was unable to induce PTEN protein levels.

Conclusions: A physiologic links exists between PTEN and IGF-II in normal mammary epithelial cells. It is possible that this represents a novel feedback loop whereby the mitogenic and antiapoptotic properties of IGF-II, mediated through PKB/Akt, are controlled in normal tissue. Loss of this feedback loop may increase the susceptibility of mammary tissue to IGF-II-induced tumorigenesis.

Introduction

Factors affecting mammary epithelial proliferation can be studied in vivo by examining mouse mammary tissue during ductal morphogenesis. This process starts during embryogenesis as a rudimentary ductal tree emanating from the nipple area (reviewed in [1]). Mammary epithelial proliferation and ductal lengthening progress slowly until around 3-4 weeks of age where an increase in ovarian estrogen secretion induces epithelial proliferation in the terminal end buds (TEBs) and increases the rate of ductal elongation [1]. Lengthening of the mammary ducts continues until the end of the fat pad is reached at which time the TEBs disappear and lengthening ceases [1]. A number of factors in addition to estrogen have been shown to participate in this developmental process such as progesterone [2-4], prolactin [3-6], epidermal growth factor [7-9], fibroblast growth factor [10] and transforming growth factor-β [11,12] and growth hormone [3,4,13].

More recent studies have demonstrated that growth hormone does not directly effect mammary morphogenesis rather it stimulates the production of insulin-like growth factor (IGF), presumably by the stromal compartment. Both IGF-I and IGF-II have potent mitogenic effects on various cell types in vitro (reviewed in [14-21]) and have been shown to promote mammary epithelial proliferation and proliferation of breast cancer cell lines [22-27]. IGFs elicit their mitogenic effects by binding to the IGF-I receptor (IGF-IR) on the surface of mammary epithelial cells and inducing signal transduction through this tyrosine kinase receptor. In vivo, IGF-I can by itself, promote ductal lengthening and act synergistically with estrogen during mammary development. The requirement of IGFs for normal ductal development has been demonstrated in IGF-I knockout mice where it was shown that mammary development is significantly reduced in these mice as measured by the number of TEBs and the percentage of the mammary gland occupied by epithelial ducts [28]. Further, estrogen was incapable of stimulating mammary development in the absence of IGF-I thus demonstrating the importance of IGF-I during this process.

The signaling pathways utilized by the IGFs to regulate mammary epithelial proliferation are not completely understood. A number of intracellular signaling molecules can be activated following IGF-IR stimulation. Two of the best-characterized IGF-IR docking proteins are Shc and insulin receptor substrate (IRS)-1. Induction of the Shc pathway leads to Erk1/Erk2 phosphorylation and activation of downstream transcription factors that regulate cell cycle progression [20,29,30]. Phosphorylation of IRS-1 leads to PI-3 kinase activation and phosphorylation of phosphatidyl inositol phosphate (PIP)-2 to PIP-3. PIP-3 promotes translocation of PKB/Akt to the plasma membrane where it can be phosphorylated by PDK-1. PKB/Akt can then phosphorylate Bad and at least in human tissue, caspase 9, and inhibit apoptosis. Recently, PKB/Akt has also been shown to promote cell cycle progression through its ability to activate p70 S6 kinase and inhibit GSK-3-mediated degradation of cyclin D1[31-33].

One of the primary regulators of PKB/Akt activity is PTEN. PTEN is a lipid phosphatase capable of decreasing PIP-3 levels and thus inhibiting translocation of PKB/Akt to the plasma membrane [34]. The ability of PTEN to regulate PKB/Akt signaling is conserved in other species including *drosophila* [35,36] and *C. elegans* [37-40]. The importance of this negative regulation of PKB/Akt phosphorylation by PTEN has been shown in PTEN heterozygous deficient mice. Loss of even one PTEN allele

in mice leads to PKB/Akt hyperactivity and the development of tumors in the endometrium, breast, prostate, gastrointestinal tract, adrenal gland, thyroid and thymus [41,42]. Further, a considerable number of reports show that tumorigenesis in humans is frequently associated with loss of heterozygosity or mutation of the PTEN gene [43].

Since the effect of IGF-II on mammary epithelial proliferation and signaling molecules utilized in vivo have not been examined we investigated the effect of transgenic IGF-II overexpression on mammary epithelial proliferation during ductal morphogenesis. We found that unlike its actions in vitro, IGF-II overexpression inhibited epithelial proliferation and ductal morphogenesis in vivo. The decrease in epithelial proliferation appeared to be regulated by a significant induction of PTEN protein in the transgenic mammary tissue with subsequent reductions in PKB/Akt phosphorylation and cyclin D1 protein levels. Direct administration of recombinant IGF-II to wild type mammary tissue resulted in an 18-fold induction in PTEN mRNA suggesting that IGF-II regulates PTEN expression. Our observations suggest that a physiologic link exists between IGF-II and PTEN in mammary epithelial cells whereby IGF-II induces PTEN expression to regulate the PKB/Akt-mediated mitogenic and antiapoptotic effects of IGF-II.

Results

Transgenic Overexpression of IGF-II Delays Mammary Ductal Morphogenesis.

To examine the effect of IGF-II overexpression on mammary ductal morphogenesis whole mount analysis was performed on wild type and MMTV-IGF-II transgenic mice. Mammary overexpression of IGF-II significantly inhibited ductal morphogenesis ($p < 0.05$) in two independent MMTV-IGF-II transgenic lines designated MI1 and MI12 (Fig. 1a-c). An average reduction of 28% in epithelial ductal length and of 25% in the number of ducts occurred at day 55 of development. Further examination revealed that ductal retardation existed at ages 42, 55 and 75 days, but not at 35 days (data not shown). At day 75, the number of terminal end buds (TEBs) remaining in the transgenic mammary tissue was significantly elevated (Fig. 1d,e) indicating that lengthening of the ducts had not been completed. These observations suggested that IGF-II overexpression affected mammary morphogenesis during pubertal development and not prior. Since growth of the ductal tree is dependent on epithelial proliferation we measured this parameter in the IGF-II overexpressors. The percentage of proliferating mammary epithelial cells was reduced 1.8-fold ($p < 0.05$) in transgenic compared to wild type mammary tissue (Fig. 1f-h). In addition, epithelial proliferation was 1.5-fold lower in transgenic TEBs that constitute the leading edge of growing ducts.

Given that IGFs are generally considered mitogenic [44], our observations were unexpected. To ensure that inhibition of ductal morphogenesis was a result of IGF-II overexpression, four additional tests were preformed. First, transgenic IGF-II overexpression was confirmed during mammary development. In situ hybridization localized far greater IGF-II RNA in transgenic versus wild type mammary epithelium from 55-day-old mice (Fig. 1i,j). Next, we measured the serum levels of 17β-estradiol and progesterone, as pubertal mammary development is dependent on these two steroid hormones. These levels remained unaltered in the MMTV-IGF-II transgenic mice compared to wild types (data not shown). Then, to determine whether epithelial IGF-II overexpression was sufficient to delay ductal progression, mammary transplants were performed. Cleared mammary fat pads were generated in both wild type and transgenic mice by removing mammary epithelial cells prior to puberty. Transgenic mammary epithelium was implanted into wild type cleared fat pads (Fig. 1l), while wild type mammary tissue was implanted into cleared transgenic fat pads (Fig. 1k). Epithelial ducts emanating from the transgenic transplants were on average 31% ($p < 0.05$) shorter than those originating from wild type transplants. Thus, epithelial IGF-II, rather than the host environment was responsible for the mammary phenotype. Finally, a biochemical approach was used to elevate mammary IGF-II. A Slow-release recombinant IGF-II pellet was implanted in the 4^{th} inguinal mammary gland of wild type mice (Fig. 1n) while the contralateral gland received a control pellet (Fig. 1m). The IGF-II pellet retarded mammary ductal length by an average of 26%. Together, these four studies established that elevated IGF-II inhibits mammary ductal morphogenesis.

Transgenic IGF-II Overexpression Elevates Mammary PTEN Protein Levels

To identify the signaling pathways responsible for the anti-proliferative properties of IGF-II, several molecules downstream of the type I IGF receptor (IGF-IR) were examined. Initially we focused on the PKB/Akt pathway since PKB/Akt activation was

recently reported to regulate cell cycle progression [31-33]. A significant reduction (77%, $p < 0.01$) in the levels of phosphorylated PKB/Akt was found in the transgenic mammary tissue (Fig. 2a,b). This finding was intriguing considering the binding of IGF-II to the type I IGF receptor (IGF-IR) normally leads to phosphorylation of PKB/Akt. We reasoned that in the transgenic mammary tissue, either activation of IGF-IR by IGF-II was abrogated or the activity of a molecule responsible for modulating PKB/Akt phosphorylation, such as PTEN, was altered. IRS-1 phosphorylation was used as an indicator of IGF-IR activity because it is phosphorylated by the IGF-IR, and it in turn leads to PI3-kinase and PKB/Akt phosphorylation [20,45]. IRS-1 phosphorylation levels were elevated in the transgenic tissue providing evidence for IGF-IR activation (Fig. 2a). Next we examined PTEN, since it inhibits PKB/Akt phosphorylation by opposing the conversion of PIP-2 to PIP-3 by PI-3 kinase [34]. The levels of PTEN protein were significantly ($p < 0.05$) elevated in the transgenic tissue (Fig. 3a,c). Specifically, PTEN was 1.8-fold higher on average in the two transgenic lines compared to wild type tissue. In addition, immunohistochemical analyses using antibodies specific for phosphorylated PKB/Akt and PTEN proteins identified that transgenic mammary epithelium had reduced phosphorylated PKB/Akt and elevated PTEN protein level (Fig. 2d-g). Moreover, the levels of cyclin D1 (Fig. 2a) were significantly reduced in the transgenic mammary tissue suggesting that the diminished levels of phosphorylated PKB/Akt in this tissue permitted more cylcin D1 degradation.

We tested whether other signaling pathways implicated in cell proliferation, namely Erk1/Erk2, p38 MAPK, JNK/SAPK, played a role in mediating the effects of IGF-II on mammary epithelial proliferation. There were no significant differences in the levels of phosphorylated Erk1/Erk2 or p38 MAPK in transgenic versus wild type mammary tissue at day 55 (Fig. 2h). As we were unable to detect phosphorylated JNK/SAPK in the mammary tissue, we assessed ATF-2, which is downstream of JNK/SAPK. The phosphorylation status of ATF-2 was also unaltered in the transgenic mammary tissue (Fig. 2h). Thus, we ruled out the involvement of these classical pathways in mediating the inhibitory effect of IGF-II on epithelial proliferation, and narrowed the IGF-II effects to the PKB/Akt pathway. Together, our data show that chronic elevation of mammary IGF-II in the MMTV-IGF-II transgenics induces PTEN protein, restricts PKB/Akt activity, reduces cylcin D1 levels and subsequently inhibits epithelial proliferation and ductal development. Whether other kinases and phosphatases are also altered remains open.

Recombinant IGF-II Induces PTEN mRNA Expression in Vivo

As an alternative means to elevate IGF-II levels, mini-osmotic pumps containing vehicle or recombinant IGF-II were implanted into wild type mice. These pumps release a constant amount of IGF-II over a 14-day period. When PKB/Akt and PTEN were examined we found that the levels of phosphorylated PKB/Akt were significantly reduced while the levels of PTEN were significantly elevated in the mammary tissue of mice receiving a pump containing recombinant IGF-II compared to those mice receiving a pump containing only vehicle (Fig. 3a). These results confirm the MMTV-IGF-II transgenic findings in that sustained elevation of IGF-II leads to decreased phosphorylated PKB/Akt and increased PTEN protein levels.

To determine whether PTEN gene responds directly to IGF-II, recombinant IGF-II was injected into wild type mouse mammary glands and PTEN mRNA levels assessed.

Dramatic transient increases in the levels of phosphorylated PKB/Akt occurred within 30-60 minutes of IGF-II treatment, confirming that injected IGF-II elicited an immediate cellular response in vivo (Fig. 3b). The IGF-II administration increased PTEN mRNA levels within 4 hours, with a highly significant 18-fold induction ($p<0.000001$) at 8 hours (Fig. 3c). Thus, IGF-II induces PTEN expression.

We also investigated whether the regulation of PTEN was specific to IGF-II. To address specificity, IGF-I or insulin were also injected into wild type mouse mammary glands. Western analysis for the levels of phosphorylated PKB/Akt indicated that IGF-I and insulin induced a similar level of PKB/Akt activation as IGF-II (Fig. 4a). However, IGF-I and insulin are only about half as effective as IGF-II in elevating the levels of PTEN mRNA (Fig. 4b). Therefore, IGF-I and insulin can also regulate PTEN expression but they are not as effective as IGF-II. In addition, mammary tissue from transgenic mice that overexpress the wild type ErbB2 receptor (MMTV-neu) was examined. The day-55 MMTV-neu mammary tissue had elevated levels of phosphorylated PKB (2.2-fold) and reduced levels of PTEN (2.5-fold) compared to wild type controls (Fig. 4c) indicating that increased ErbB2 signaling did not lead to elevated PTEN levels. Therefore, regulation of PTEN levels in the mammary gland is restricted to specific receptor tyrosine kinases that includes the IGF-IR and/or insulin receptor.

Discussion

We have used the process of mammary ductal morphogenesis and MMTV-IGF-II transgenic mice to examine the effects of IGF-II overexpression on mammary epithelial proliferation in vivo. This system permits the analysis of chronic IGF-II elevation on mammary epithelial cell proliferation including dissection of the signal transduction pathways in the context of the appropriate hormone and growth factors milieu, a scenario that cannot be recapitulated in tissue culture experiments. Although IGF-II is a potent mitogen for mammary epithelial cells in culture, we demonstrate here that under certain physiological conditions, elevated levels of IGF-II significantly reduced epithelial proliferation resulting in delayed mammary ductal morphogenesis during pubertal development. IGF-II overexpression induced an elevation in PTEN protein, presumably leading to decreased PKB/Akt phosphorylation, cyclin D1 protein levels and cell proliferation.

IGF-II Overexpression Delays Mammary Morphogenesis by Inhibiting of Epithelial Proliferation. Our results demonstrate that overexpression of IGF-II inhibited, rather than stimulated mammary morphogenesis during pubertal development. This inhibitory effect of IGF-II was observed in MMTV-IGF-II transgenic mice and confirmed through implantation of pellets containing recombinant human IGF-II into developing mammary glands of wild type mice. In addition, transplanting transgenic mammary tissue into cleared fat pads of wild type mice showed that overexpression of IGF-II and not alterations in hormonal or stromal conditions in the MMTV-IGF-II transgenics was responsible for the delay in ductal morphogenesis. The delayed lengthening of epithelial ducts in transgenic mammary tissue was attributed to a significant reduction in epithelial proliferation. Since epithelial proliferation in TEBs is pivotal for ductal elongation, this reduction in proliferation, at least in part, explains the delay in mammary development in the IGF-II overexpressors.

This finding is intriguing considering that IGFs are potent mitogens for mammary epithelial cells and breast cancer cell lines in vitro [22-27]. One property of tumor cells and established cell lines is that certain proliferative controls have been lost. It is possible that immortalized and tumor cell lines, derived from mammary tissue, have deficiencies signaling pathways that regulate antiproliferative molecules such as PTEN.

Based on the literature available it is difficult to address whether overexpression of IGF-I also inhibits pubertal mammary ductal morphogenesis. Implantation of IGF-I pellets or systemic IGF-I treatments in estradiol-treated, hypophysectomized, gonadectomized, immature male rats promoted mammary development [46]. However, the aforementioned studies represent artificial conditions to study the effect of IGFs on mammary epithelial proliferation. The only other study to examine the effect of IGFs on pubertal female mammary development in vivo was performed by Weber et al. [47]. In this study, MMTV-IGF-I transgenic mice were found to have enhanced lobulo-alveolar development in 50 day-old mice that was not observed in wild type controls. Although an increase in lobulo-alveolar development would suggest elevated amounts of epithelial proliferation [48], this property was not assessed. In addition, the effects of IGF-I overexpression on ductal lengthening or TEB formation were not examined. Therefore, overexpression of IGF-I can induce precocious mammary development

however its effect on ductal elongation during female pubertal mammary development remains unclear.

Interestingly we have also found that overexpression of the ErbB2 receptor, although associated with increased proliferation and mammary tumorigenesis [49,50], significantly inhibited mammary ductal development (unpublished observations and [51]). Therefore, it appears that inappropriate expression of mitogenic signals may deregulate proliferative processes resulting in reduced proliferation. Support for this idea stems from studies in the C/EBPbeta null mice. Loss of the C/EBP gene induced an increase in progesterone receptor expression in the mammary tissue and altered the distribution from a nonuniform to a uniform pattern of expression [52]. This alteration in progesterone receptor level and expression pattern inhibited epithelial proliferation and lobulo-alveolar development although the progesterone receptor is normally required for this process. Therefore, inappropriate expression of genes normally involved in stimulating proliferation can under certain physiological conditions, inhibit proliferation.

The antiproliferative effects of IGF-II were limited to the development stage as proliferation and differentiation during gestation was unaffected. We examined mammary tissue from wild type and MMTV-IGF-II transgenic mice on days 4.5, 8.5, 12.5, 14.5, 16.5, and 18.5 of gestation and found no differences at the whole mount or histological level. Further, the percentage of epithelial cells proliferating was similar in transgenic and wild mammary tissue at all gestational stages.

Inhibition of Epithelial Proliferation and Ductal Morphogenesis in MMTV-IGF-II Transgenic Mice is Associated with Elevated Levels of PTEN. To identify the pathways involved in mediating the growth inhibitory signals from IGF-II, several signal transduction molecules downstream of the IGF-IR were investigated. We examined the phosphorylation status of Erk1/Erk2 and p38 MAPK proteins and found no significant difference in the levels between wild type and transgenic mammary tissue suggesting that these proliferative pathways were not affected by IGF-II overexpression. The JNK/SAPK pathway was also examined but phosphorylated JNK/SAPK could not be detected in developing mammary tissue. A potential target of the JNK/SAPK pathway, the ATF-2 transcription factor, was also examined and its phosphorylation status was not significantly different in transgenic mammary tissue indicating that this pathway did not mediate the antiproliferative signals.

We did however observe a significant reduction in the levels of phosphorylated PKB/Akt in both of the MMTV-IGF-II transgenic lines examined. PKB/Akt is an antiapoptotic molecule whose properties have been well documented (reviewed in [53-56]). Activation of a number of receptors, including IGF-IR stimulates PI3-kinase activity and subsequent phosphorylation of PKB/Akt. Once phosphorylated PKB/Akt has been proposed to inhibit the proapoptotic molecules Bad and caspase 9. Therefore, the decrease in phosphorylated PKB/Akt in the transgenic mammary tissue would suggest that mammary development was delayed due to a higher rate of epithelial apoptosis. However, we were unable to detect significant levels of apoptosis in the developing transgenic mammary tissue using either in situ end labeling or Hoescht staining. This does not rule out the possibility that the transgenic epithelial cells are more susceptible to apoptotic stimuli.

More recently, the activation of PKB/Akt has also been shown to stimulate proliferation. D-type cyclins are synthesized during the G_1 phase of the cell cycle and become complexed with cyclin D-dependent kinases (reviewed in [57,58]). These complexes cause phosphorylation of the retinoblastoma protein and thus stimulate proliferation. Proliferation is halted through phosphorylation of cyclin D1, which targets this protein for degradation. Diehl et al [59] have shown that GSK-3β is one of the molecules that can phosphorylate cyclin D1 and promote its degradation. Since GSK-3β activity can be inhibited by PKB/Akt-dependent phosphorylation, PKB/Akt promotes cell proliferation by stabilizing cyclin D1 protein levels [31]. In light of the fact that cyclin D1 levels are significantly decreased in our transgenic mammary tissue it appears that IGF-II overexpression inhibits epithelial proliferation by reducing the levels of phosphorylated PKB/Akt and cyclin D1 protein.

The decrease in the level of phosphorylated PKB/Akt despite the high levels of phosphorylated IRS-1 lead us to examine the levels of PTEN. Translocation of PKB/Akt and its subsequent phosphorylation is dependent on PI3-kinase-mediated phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 [34]. Thus, elevated levels of PTEN could dephosphorylation PtdIns(3,4,5)P_3 and thus prevent PKB/Akt phosphorylation. When the levels of PTEN protein were examined we observed a significant increase in PTEN levels in the transgenic mammary tissue compared to wild-type tissue. The fact that the changes in phosphorylated PKB/Akt and PTEN levels were a result of IGF-II overexpression was confirmed using mini-osmotic pumps. Decreased levels of phosphorylated PKB and increased levels of PTEN were also observed in mammary tissue from wild type mice implanted with a mini-osmotic pump containing recombinant IGF-II compared to mice implanted with vehicle-containing pumps. Thus, sustained elevation in the levels of IGF-II leads to an increase in PTEN protein and a decrease in phosphorylated PKB/Akt.

IGF-II Regulates PTEN Expression

Factors that regulate PTEN expression and protein levels are poorly understood. TGF-β has been shown to reduce PTEN mRNA in a human keratinocyte tumor cell line but the physiological significance of this finding is unclear [60]. In addition, a correlation between PTEN RNA levels and progesterone in human endometrial tissue has been reported [61]. Our data indicates that PTEN expression is regulated by IGF-II and this interaction affects mammary epithelial proliferation during mammary ductal morphogenesis. Regulation of PTEN by IGF-II was evident in MMTV-IGF-II transgenic mice, wild type mice receiving IGF-II containing mini-osmotic pumps and mammary tissue receiving direct administration of recombinant IGF-II. The ability of IGF-II to regulate PTEN expression is somewhat specific in that IGF-II was more effective than IGF-I or insulin in regulating PTEN expression and overexpression of the ErbB2 receptor did not lead to elevated levels of PTEN. Therefore, regulation of PTEN may be restricted to a subset of receptor tyrosine kinase receptors that includes the IGF-IR and insulin receptor but not ErbB2 receptor.

Interactions between PTEN and insulin or IGF signaling have been reported. PTEN has been shown to increase IRS-2 levels [62] and inhibit IRS-1 activity [63]. It has been proposed that PTEN is part of a feedback mechanism where the levels of PIP-3 regulate the amount of IRS-2 available for insulin signaling [62]. Our results suggest

that a feedback mechanism between IGF-II and PTEN also exists whereby increased signaling downstream of the IGF-IR initially induces PKB/Akt activation followed by increased PTEN expression to regulate the proliferative and antiapoptotic effects of IGF-II mediated through the PKB/Akt pathway.

If such a feedback loop does exist it would have important implications in IGF-II-mediated mammary tumorigenesis. In normal tissue, any increase in IGF-II signaling, especially PKB/Akt phosphorylation, would be balanced by an increase in PTEN expression. However, loss of the link between IGF-II and PTEN would permit uncontrolled activation of PKB/Akt in response to elevated IGF-II levels possibly resulting in the development of mammary tumors. The existence of an intact feedback loop may explain the low frequency of mammary tumors observed in our MMTV-IGF-II transgenic mice as increased mammary IGF-II signaling is compensated by elevated levels of PTEN protein. It is intriguing that the mammary tumors that have developed in the MMTV-IGF-II transgenic mice have reduced levels of PTEN mRNA compared to non-tumor bearing transgenic mammary tissue as well as to wild type mammary tissue (unpublished observations). Another interesting observation is that although ErbB receptors activate many of the same intracellular signaling molecules (i.e. PKB/Akt, Erk1/Erk2 and p38 MAPK) as the IGF-IR, overexpression of the ErbB2 receptor did not induce PTEN levels. The lack of PTEN upregulation may partially explain why MMTV-ErbB2 transgenic mice frequently develop mammary tumors [49] while mammary tumors in MMTV-IGF-II transgenics are rare. The importance of PTEN in regulating IGF-II mammary tumorigenesis requires further investigation.

The IGF system and the tumor suppressor gene, PTEN, have thus far been independently implicated in the generation of human cancers. We have discovered a novel link between these molecules whereby IGF-II modulates PTEN protein levels to control signaling through the PKB/Akt pathway, during the developmental event of mammary morphogenesis. Loss of the IGF-II-PTEN link, however, may be one of the events required prior to the induction of mammary tumors by IGF-II.

Methods

Mice

MMTV-IGF-II mice have been described [64]. All mice were maintained at the Ontario Cancer Institute animal facilities, following the guidelines established by the Canadian Council on Animal Care.

Whole Mount Analysis, *in situ* hybridization and immunohistochemistry. Whole mount analysis [65], in situ hybridization [66,67], and bromodeoxyuridine (BrdU) immunohistochemistry [68] were performed as previously described. For PTEN and phosphorylated PKB/Akt immunohistochemistry 4% buffered formalin-fixed, paraffin-embedded sections were dehydrated and incubated for 16 min at 95-100°C using microwave antigen retrieval technique. Sections were then incubated with either anti-PTEN (FL-403, Santa Cruz Biotech; 1:200 dilution) or anti-phosphorylated PKB/Akt (Ser473) (NEB; 1:50 dilution). The binding of primary antibodies was visualized using peroxidase-conjugated anti-rabbit or mouse IgG. All sections were counter-stained with haematoxylin.

Serum Progesterone and 17-β-Estradiol Levels. Approximately 600-800μl of blood was removed from the mouse at sacrifice. Clotting was permitted for 30 min at room temperature and serum separated by centrifuging the samples at 6000-x g for 30 min. Serum samples were stored at -70°C until the samples were analyzed for progesterone and 17-β-estradiol by Dr. S. Tokmakejian (University of Western Ontario, Canada) using previously described protocols [69].

Elvax-40 Slow Release Pellets. Elvax-40 pellets containing recombinant human IGF-II were generated as previously described [68]. Pellets containing 300 ng of rhIGF-II were implanted in the 4th-inguinal mammary gland of wild-type mice at 33 days of age. Control pellets containing only the vehicle (PBS) were implanted in the contralateral mammary gland. The mice were sacrificed 7 days later and the 4th-inguinal mammary glands analyzed.

Mammary Transplants. The 4th inguinal fat pads of 21 day-old anesthetized wild type and transgenic mice were cleared by removal of the mammary tissue between the lymph node and the nipple area. An approximate 2mm x 2mm piece of this removed tissue was then transplanted such that transgenic mammary tissue was transplanted into the cleared fat pad of wild type mice and wild type mammary tissue was transplanted into the cleared fad pad of transgenic mice. Transplants and unmanipulated 4th inguinal mammary glands were removed 21 days later and subject to whole mount analysis.

Implantation of Mini-Osmotic Pumps. Osmotic pumps (Durect Corp., Cupertino, CA) were filled with 40ug of recombinant IGF-II (Calbiochem, La Jolla, CA) in 100ul of sterile PBS containing 0.1% BSA or PBS containing 0.1% BSA only. The osmotic pumps had a pumping rate of 0.25μl/hr and would pump at this rate over a 14-day period. Pumps were surgically implanted in the abdominal cavity of 5-week old wild type female FVB

mice and mammary tissue was removed for analyses 14 days later. Detection of PKB and PTEN were performed as described above.

Injection of Recombinant Proteins. Human recombinant IGF-II (Calbiochem, La Jolla, CA) was resuspended in sterile PBS containing 0.1% BSA at a concentration of 100ng/μl. Recombinant IGF-I (Calbiochem) and insulin (Sigma) were resuspended in 10mM acetic acid containing 0.1% BSA and 5mM HCl, respectively. Mice were anesthetized and the 4th inguinal mammary glands were exposed surgically. Ten microlitres of protein (1μg IGF-II, 1μg IGF-I, or 10μg insulin) or appropriate vehicle were injected into the mammary gland and the incisions were closed. Mice were then sacrificed 0.5, 1, 2, 4, and 8 hours after the protein injection and the mammary tissue was harvested for western analysis or RT-PCR as described above.

Western Analysis. Protein isolation and western blotting was performed as described [64]. Primary antibodies for Akt, phosphorylated Akt (Ser473), Erk1/Erk2, and phosphorylated Erk1/Erk2 (Thr202/Tyr204) (New England Biolabs, Beverly, MA) were used at a dilution of 1:1000 in 5% skim milk in TBST while antibodies for p38 MAPK, phosphorylated p38 MAPK (Thr180/Tyr182), ATF-2, phosphorylated ATF-2 (Thr71), and PTEN (New England Biolabs, Beverly, MA) were used at a dilution of 1:1000 in 5 % BSA in TBST. The primary antibodies for phosphorylated IRS-1 (Ser612) (Medicorp, Montreal, QU, Canada) and IRS-1 (Upstate Biotechnology, Lake Placid, NY) were used at dilutions of 1:650 and 1:1000 in 5 % skim milk respectively. Membranes were incubated with the appropriate antibody overnight at 4°C. Proteins were detected using HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and LumiGLO reagents (New England Biolabs, Beverly, MA) and were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA). Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C. Protein loading was determined by staining membranes in 1 % amido black in 50 % methanol/10 % glacial acetic acid for 20 min.

RT-PCR. RNA was extracted from mammary tissue using the method of Chomczynski and Sacchi [70]. RNA (2 μg) was reverse transcribed using Superscript II reverse transcriptase (Gibco BRL, Burlington, ON, Canada). The resulting cDNAs were amplified using a 56°C annealing temperature and 23 cycles with primers for either PTEN (5'-ACAGACCTAGGCTACTGCTC-3' and 5'-CTAGAAGCAAGACTTCCGTTC-3'), or the house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) (5'-GTTGGATACAGGCCAGACTTTGTTG-3' and 5'-GATTCAACTTGCGCTCATCTTAGGC-3'). PCR samples were electrophoresed through a 2% agarose gel, transferred to nylon membrane and probed with either a [^{32}P]-dCTP-labeled PTEN or a [^{32}P]-dCTP-labeled HPRT probe. Membranes were developed in phosphoimager cassettes and the radioactive signal from PTEN was quantified relative to that of HPRT using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Statistics. All values are presented as mean ± SEM. Statistical significance was determined using the Student's t test and values were considered significant when $p < 0.05$.

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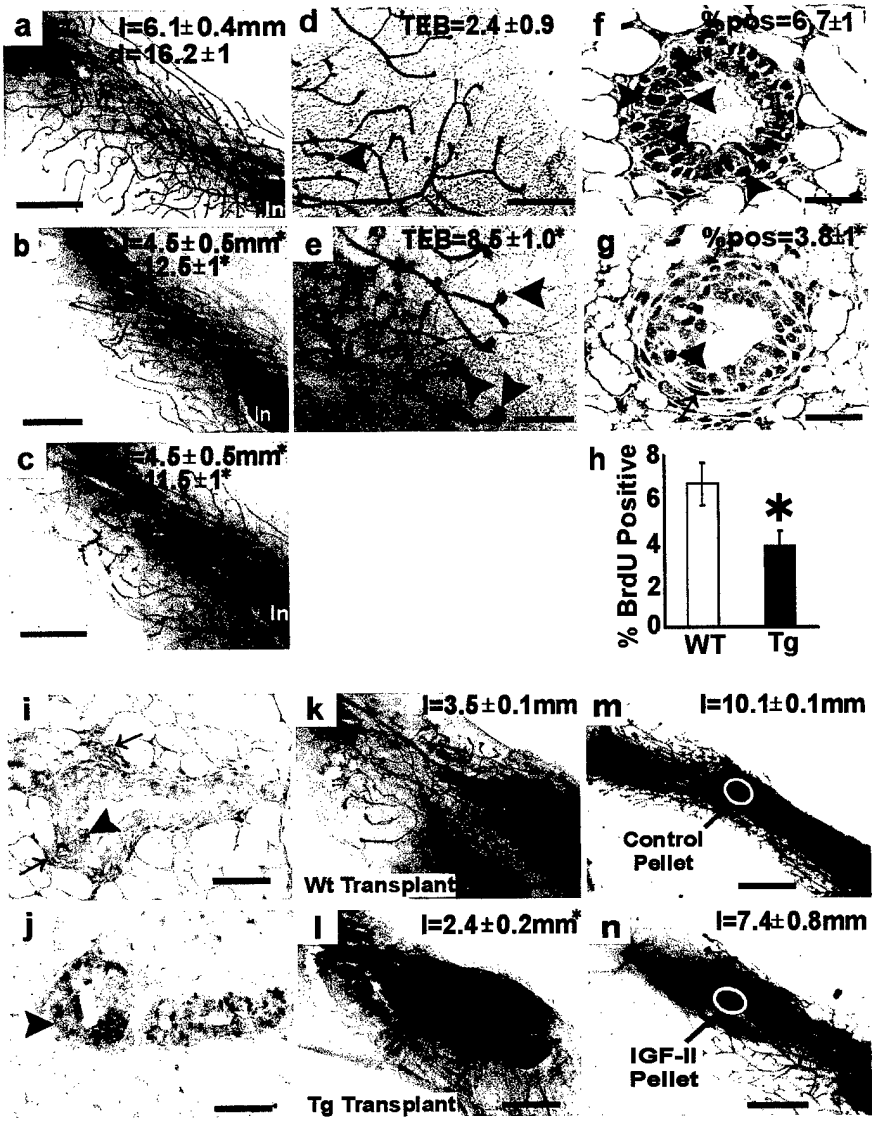
Figure Legends

Figure 1. Decreased mammary morphogenesis and epithelial proliferation in MMTV-IGF-II transgenic mice. Carmine-alum-stained wholemounts of (**a**) wild type, (**b**) MMTV-IGF-II transgenic line MI1, and (**c**) transgenic line MI12 at day 55 of development. The average length (l) of the epithelial ducts extending beyond the lymph node (ln) and number of ducts (d) are provided in the upper right corner, * $p < 0.05$, $n \geq 4$, size bars, 1mm. Representative wholemount of (**d**) wild type and (**e**) transgenic 75-day old mammary tissue. The number of terminal end buds (TEBs) are provided in the upper right corner (* $p < 0.05$, $n \geq 4$) and identified by arrowheads, size bars 400μm. Mammary epithelial proliferation was determined by BrdU immunohistochemistry in (**f**) wild type (n=4) and (**g**) transgenic tissue (n=8) at day 55 of development, size bars, 75μm. Arrowheads denote BrdU positive epithelial cells. The percentage of BrdU positive epithelial cells is provided in the upper right corner and presented in the bar graph (**h**), * $p < 0.05$. In situ hybridization for IGF-II in (**i**) wild type and (**j**) transgenic mammary epithelial ducts and terminal end buds, size bars, 50μm. Wholemount analysis of (**k**) wild type epithelial cells transplanted into a cleared transgenic fat pad and (**l**) transgenic epithelial cells transplanted into a cleared wild type fat pad, size bars, 1mm. Average length of epithelial ducts from the center of the transplant extending towards the end of the fat pad are provided in the upper right corner, * $p < 0.05$, n=3. Wholemount analysis of wild type mammary glands containing (**m**) a control pellet or (**n**) a pellet containing IGF-II, size bars, 1mm.

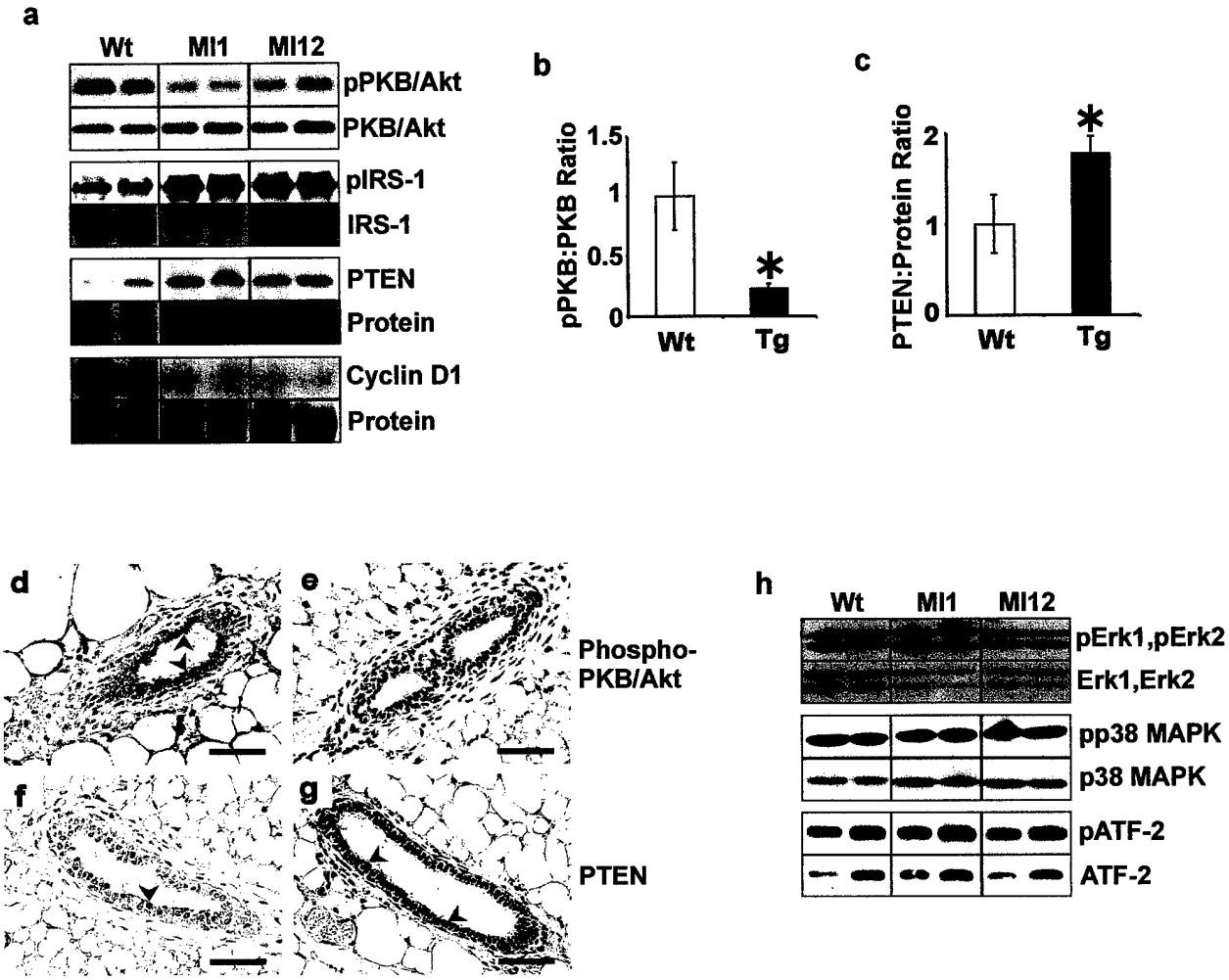
Figure 2. Decreased levels of phosphorylated PKB/Akt and elevated levels of PTEN in MMTV-IGF-II transgenic mammary tissue. Western analysis of (**a**) PKB/Akt, IRS-1, PTEN, and cyclin D1 in developing wild type and transgenic mammary tissue. Quantification of the relative levels of (**b**) phosphorylated PKB/Akt and (**c**) PTEN in wild type and transgenic tissue, * $p < 0.05$, n=4. Immunohistochemistry for (**d,e**) phosphorylated PKB/Akt and (**f,g**) PTEN in (**d,f**) wild type and (**e,g**) MMTV-IGF-II mice, size bars 50μm. Arrowheads indicate signal specific for phosphorylated PKB/Akt or PTEN. **h,** Western analysis of Erk1/Erk2, p38 MAPK, and ATF-2 in wild type and transgenic mammary tissue.

Figure 3. IGF-II induces PTEN expression. (**a**) Western analysis of PKB/Akt and PTEN from mammary tissue taken from mice implanted with an osmotic pump containing either vehicle or recombinant IGF-II. The bar graph represents the relative levels phosphorylated PKB/Akt and PTEN, * $p < 0.05$, n=4. (**b**), Western analysis of phosphorylated PKB and total PKB following injection of either vehicle (V) or IGF-II (I) into wild type mammary tissue. The bar graph represents the relative levels phosphorylated PKB/Akt from two independent experiments. (**c**), RT-PCR of PTEN and HPRT in mouse mammary tissue following injection of either vehicle or IGF-II. The bar graph represents the relative normalized PTEN mRNA levels from four independent mice each at 4 and 8 hours for the IGF-II treatment and eight mice for the vehicle, * $p < 0.05$. The 4hr and 8hr vehicle injected samples were pooled since there was no significant difference between the different time points.

Figure 4. Specificity of PTEN regulation by IGF-II. **a**, Western analysis of phosphorylated PKB and total PKB following injection of either vehicle, IGF-II, IGF-I or insulin into wild type mammary tissue. The bar graph represents the relative levels phosphorylated PKB/Akt from two independent mice. **b**, RT-PCR of PTEN and HPRT in mouse mammary tissue following injection of vehicle, IGF-II, IGF-I or Insulin. The bar graph represents the relative normalized PTEN mRNA levels from three independent mice each at 8 hours after protein administration. **c**, Western analysis of pPKB/Akt, PKB/Akt and PTEN in day 55-old wild type and MMTV-neu mammary tissue. The bar graph represents the relative levels of pPKB/Akt or PTEN in the MMTV-neu mammary tissue relative to controls, * $p < 0.05$, n=4.



Moorehead et al. Fig. 1



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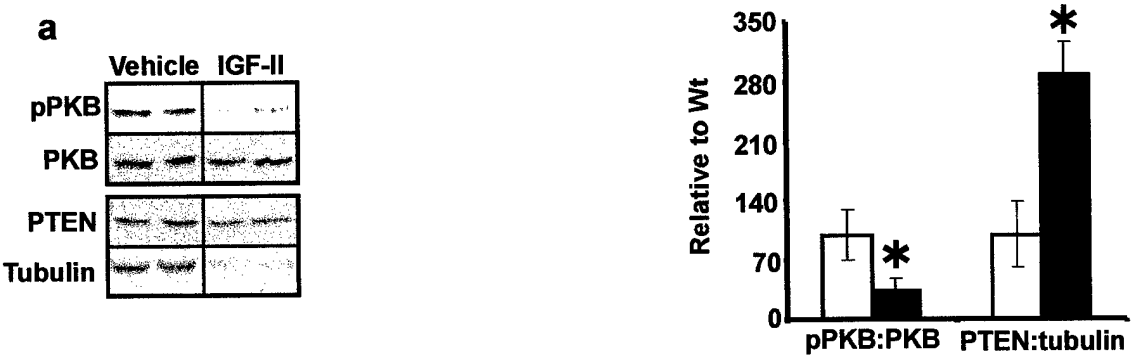
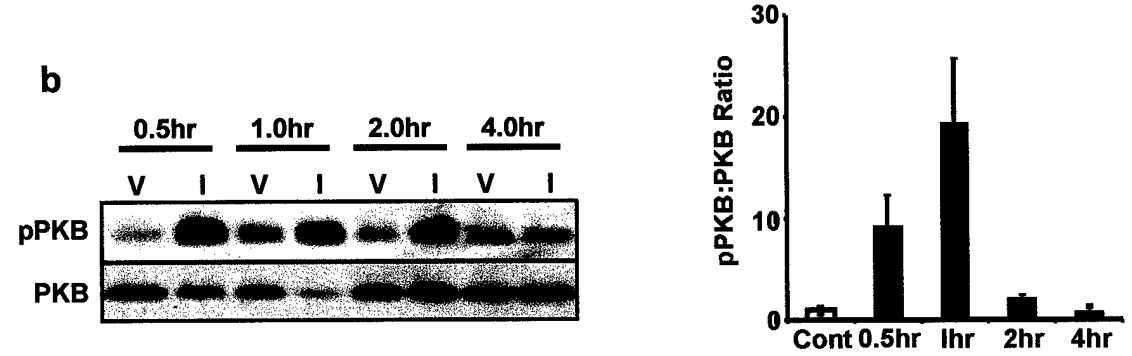
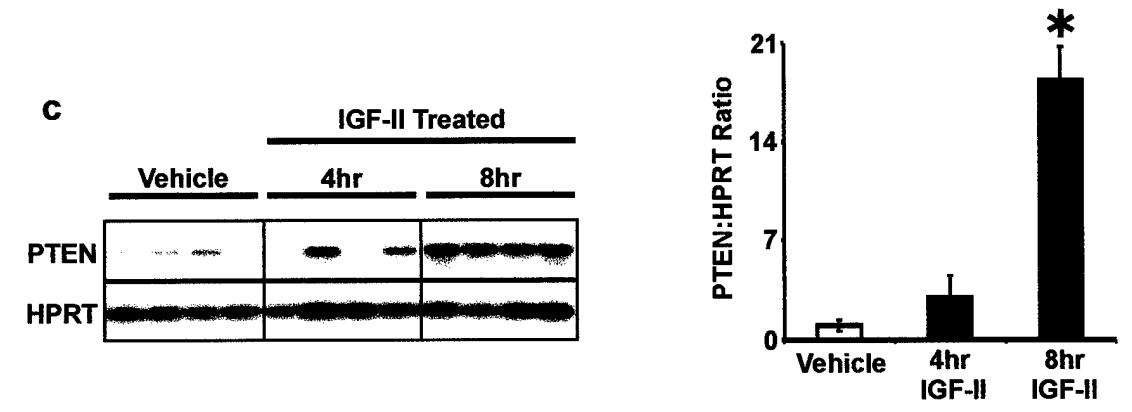
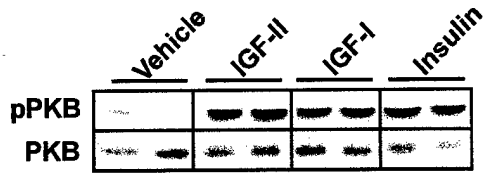






Moorehead et al. Fig. 3

a



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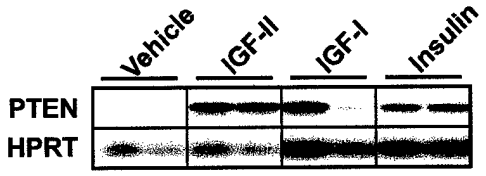
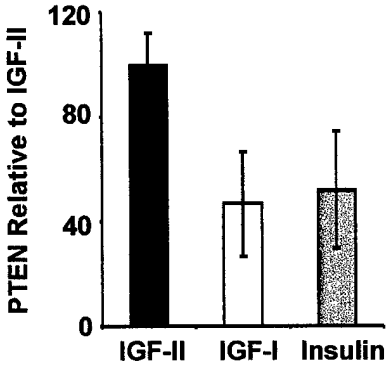
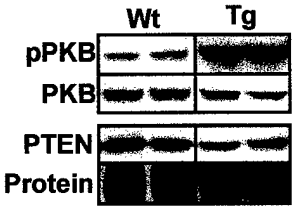
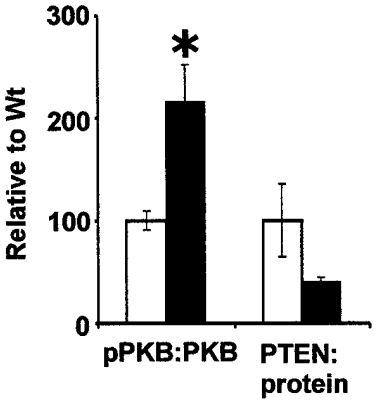




c





Moorehead et al. Fig. 4

Spontaneous IGF-II-Induced Lung Tumors Contain High Levels of Phosphorylated CREB

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Abstract

Generation of MMTV-IGF-II transgenic mice created a model where the IGF-II gene was overexpressed in a number of tissues thus permitting the investigation of whether IGF-II was causal for tumorigenesis. Histological changes were observed in the reproductive tissues, lungs, lymphatics, and lacrimal glands of the IGF-II overexpressors. Although many of the tumors maintained high levels of IGF-II RNA, tumor spectrum did not correlate with the organ-specific IGF-II expression levels. Signaling downstream of the IGF-IR was investigated to examine which pathways were altered during tumorigenesis. Our study focused on the IGF-II-induced lung tumors. We found that either the Erk1/Erk2 or p38 MAPK pathway, but not both, were activated in each of the tumors. Further investigation revealed that all of the lung tumors examined contained high levels of phosphorylated CREB suggesting that both the Erk1/Erk2 and 38 MAPK pathwaysthes converged to activate this transcription factors. Elevated levels of phosphorylated CREB were also detected in the uterine, male accessory gland and lacrimal gland hyperplasias/tumors. These data indicate that differential organ sensitivity to IGF-II-induced tumorigenesis exists. Moreover, activation of specific transcription factors, and not necessarily upstream signaling molecules, may represent a more clinically relevant event in IGF-II-induced tumorignesis.

Introduction

It is not surprising that alterations in the insulin-like growth factor (IGF) family are associated with a number of human tumors (reviewed in[1]) since IGFs can stimulate cell proliferation and inhibit apoptosis (reviewed in[2-4]), two hallmarks of tumorigenesis. The IGF family consists of two ligands, IGF-I and IGF-II, two receptors, IGF-IR, IGF-IIR, six high affinity binding proteins, IGFBP-1 to IGFBP-6 and a number of IGF-like binding proteins (reviewed in[3,5,6]). Both IGF-I and IGF-II induce intracellular signaling primarily through the IGF-IR, however, more reports of IGF-insulin receptor hybrids are appearing and their contribution to IGF signaling remains unclear[7,8]. Binding of IGF-I or IGF-II to the type I receptor induces phosphorylation of the β-chains and promotes the binding of a number of docking proteins. Two of the best-characterized IGF-IR docking proteins are Shc and IRS-1[9-11]. Activation of the Shc pathway leads to Erk1/Erk2 phosphorylation and elevated activation of downstream transcription factors that regulate cell cycle progression[12]. Activation of the IGF-IR can also lead to activation of p38 MAPK and JNK/SAPK either through Shc-dependent or -independent mechanisms[2-4]. These three pathways are thought to regulate the mitogenic effects IGFs through their actions on transcription factors and the induction of gene expression. However, these pathways are not restricted to effecting proliferation as it has been shown that Erks can also phosphorylate Bad and thus inhibit apoptosis[13,14].

Activation of IRS-1 leads to PI-3 kinase activation and phosphorylation of phosphatidylinositol (4,5)-diphosphate PIP-2 to PIP-3. PIP-3 promotes translocation of PKB/Akt to the plasma membrane where it can be phosphorylated by PDK-1[15]. Once activated PKB/Akt can phosphorylate Bad[16-18], and at least in human tissue, caspase 9 [19], leading to inhibition of apoptosis. PKB/Akt has also been shown to promote cell cycle progression through its ability to activate p70 S6 kinase and inhibit GSK-3-mediated degradation of cyclin D1[20-22].

To examine the tumorigenic potential of IGF-II and the signaling mechanisms used by this molecule to induce tumorigenesis, spontaneous tumors in MMTV-IGF-II transgenic mice were examined. These transgenic mice have been previously characterized[23]. The MMTV promoter directs expression of IGF-II to mammary epithelial cells however it also induces expression of IGF-II in a number of other organs. In addition to the mammary, we found transgene-specific expression in the kidney, uterus, ovary, liver, spleen, pancreas, and salivary (Fig. 1A). MMTV-directed expression of transgenes in many of these organs has been previously reported[24,25].

Spontaneous tissue abnormalities arise in MMTV-IGF-II transgenic mice as early as 6 months of age and 79% of the mice over one year of age display histological alterations such as hyperplasia or overt tumors. The spectrum and frequency of tissues affected is provided in Fig. 1B,C. Male transgenic mice develop alterations in the reproductive organs, lung, lymphatics, and lacrimal gland while female transgenic mice developed alterations in the reproductive tissues (ovary – 34%, uterus – 18%, and mammary – 5%), lung, and lymphatics.

Specifically, compromised lungs showed one or multiple sub-pleural well-circumscribed nodules that histologically were solid epithelial tumours in nature, not encapsulated and ranged between benign adenomas and locally invasive well-differentiated non-metastatic adenocarcinomas (Fig. 2A,B). These tumours were classified as bronchial adenocarcinomas[26].

Alterations in the male reproductive glands involved particularly the bulbourethral glands. These presented as large bilateral cystic masses located in the inguinal area with sizes of up to 2 cm in diameter. Histological analyses revealed that the walls were lined by mucinous cuboidal epithelial cells, at times forming intraluminal papillae, and constituted by connective fibrous tissue and striated bulbocavernous and ischiocavernous muscle (Fig. 2C). In some instances, the tumours were multicystic, and showed malignant characteristics including epithelial multilayering, necrosis and back-to-back gland organization discontinuously separated by thin collagenous and vascular cores. In some areas it was possible to observe invasion of the glandular tissue into adjacent striated muscle and adipose tissue, where granulomas containing cholesterol crystals were formed. The cysts were at times hemorrhagic and contained a proteinaceous glandular secretion. Overall, these findings were those of benign mucinous cystadenomas in some cases and well-differentiated non-metastatic mucinous cystadenocarcinomas of the bulbourethral glands. According to Mitsumori and Elwell[27] there are no previous published reports of these tumours developing spontaneously in mice.

Neoplasms of the female reproductive organs were found in the uterus, ovaries and mammary glands. In eight mice the uteri were enlarged and filled with partially necrotic tumours that focally infiltrated the myometrium and serosa (Fig. 2D). Histologically these tumours were epithelial in origin as documented by positive keratin and laminin immunostaining (data not shown), and contained sheets of malignant round or polygonal cells with mild to moderate atypia, low to moderate mitotic activity confirmed by BrdU incorporation, and good focal microvascular supply in some areas. Since these tumours occasionally showed PAS positive staining (data not shown) and, more rarely, pseudoglandular architecture they were classified as non-metastatic poorly differentiated endometrial adenocarcinomas. One or both ovaries in a number of mice where enlarged due to almost complete replacement of the ovarian stroma and follicles by luteal bodies (Fig. 2E). These had ill-defined borders and were formed by luteal cells that at times displayed cellular and nuclear pleomorphism and overall preserved architecture. According to Sass and Rehm[28] these alterations were classified as multinodular ovarian luteomas. One mouse developed a mammary tumour characterized by a unilateral mass that histologically invaded and destroyed adjacent striated muscle and was classified as a well-differentiated type A adenocarcinoma[29] (Fig. 2F).

Some mice developed unilateral overgrowth of the Harderian glands, evident by anterior eyeball protrusion. In these tumours, gland structure was preserved with marked increase in size. Histologically, glandular structure was preserved with occasional intraluminal papillary formations. Tumours were cytologically benign with cuboidal or cylindrical cells displaying foamy, and at times vacuolated and basophilic, mucinous cytoplasm. No mitotic figures or atypia were observed. In concordance with Sheldon [30], these spontaneous tumours were classified as acinar adenomas.

Finally, several mice developed lymphomas that infiltrated lymphoid and other organs including lungs and kidneys. Histologically and immunohistochemically these tumors were diffuse large cell non-Hodgkin's T-cell lymphomas (Fig. 2G). The cells forming these tumors displayed large round to oval nuclei with occasionally clumped chromatin, high mitotic activity and a narrow rim of basophilic agranular cytoplasm, and

were focally surrounded by rare eosinophils or mature lymphocytes. Since the tumour cells displayed cell membrane immunostaining with an anti-CD3 antibody (Fig. 2H) but not anti-B220 antibody (Fig. 2I) they were classified as lymphoblastic in origin[31].

Organ-specific IGF-II expression was investigated to examine whether the ability of IGF-II to induce histological alterations was related to the level of IGF-II expression (Fig 3A). Analysis of adult (15 week) non-tumor bearing female transgenic mice showed that transgenic IGF-II levels are highest in the salivary gland, a tissue that did not display any abnormalities. The mammary gland and lung had similar levels of IGF-II expression and these levels were at least 20-fold lower than the salivary gland (Fig. 3B). The mammary tissue rarely developed tumors while tumors were frequently observed in the lungs. The ovary and uterus had IGF-II levels that were barely detectable after quantitative RT-PCR yet alterations were common in these tissues. Thus, the induction of histological changes by IGF-II does not correlate with expression levels suggesting that certain tissues are more susceptible to IGF-II-induced alterations.

Since it is possible that the levels of IGF-II during development are more predictive of its ability to cause tissue changes, IGF-II expression was examined in developing transgenic mice (5 week). Again we observed relative expression levels of IGF-II similar to that in adult transgenic mice suggesting that organ-specific IGF-II levels during development were also unable to predict the probability of transformation (data not shown). Moreover, transgene-specific expression could be detected in both salivary and lung tissue in day 16.5 embryos indicating that tissue specific expression of the transgene during embryogenesis could not explain the difference in tissue susceptibility to IGF-II induced tumorigenesis (Fig. 3C-F).

To determine whether the spontaneous tumors in the MMTV-IGF-II mice maintained IGF-II expression, in situ hybridization was performed. We found that IGF-II RNA was detectable in a high percentage of cells found in tumors arising in the uterus, lung, and mammary (Fig. 3G-I). Other tumor sites were not examined. Therefore, it appears that IGF-II expression is maintained in the tumor tissue.

Since IGF-II was still expressed in the tumors, signaling molecules downstream of the IGF-IR were examined to assess which pathways were being utilized by these tumors. We focused on the lung tumors since this site was affected in both male and female mice and adequate tissue could be collected for protein analyses. Initially we examined the PKB/Akt pathway since we had previously shown that the phosphorylation status of this molecule is altered in the mammary tissue of the MMTV-IGF-II transgenic mice[23]. Further, elevated PKB/Akt phoshorylation can lead to increased proliferation, suppression of apoptosis and high levels of phosphorylated PKB/Akt has been associated with tumorigenesis[16,32]. We found that the levels of phosphorylated PKB/Akt were not elevated in our lung tumor tissue compared to wild type lungs suggesting that progression of IGF-II-induced tumors was not dependent on elevated PKB/Akt phosphorylation (Fig. 4a).

Next we looked at the more classical proliferative pathways downstream of the IGF-IR, namely Erk1/Erk2 and we found that two of the lung tumors (T2 and T3) had elevated levels of phosphorylated Erk1/Erk2 while the other three did not (Fig. 4b). To further explore signaling downstream of the IGF-IR we also investigated the levels of phosphorylated p38 MAPK and JNK/SAPK. We found that the three tumors (T1, T4 and T5) that did not have elevated levels of phosphorylated Erk1/Erk2 had elevated

levels of phosphorylated p38 MAPK (Fig. 4c). The levels of phosphorylated JNK/SAPK were barely detectable but did not appear to differ between the lung tumor tissue and wild type lungs (data not shown). Since some of the transcription factors activated by Erk1/Erk2 and p38 MAPK overlap we examined two targets that are common to these upstream signaling proteins, Elk-1 and CREB to assess whether IGF-II was utilizing two independent signaling pathways or whether these pathways converged on common transcription factors. We were unable to detect phosphorylated Elk-1 in any of the samples however the levels of phosphorylated CREB were elevated in all 5 of the lung tumor samples compared to the wild type tissue (Fig. 4d). The anti-phosho-CREB antibody also recognizes phosphorylated ATF-1 and the levels of this phosphorylated transcription factor were also elevated in all of the lung tumor samples (Fig. 4d). The level of phosphorylated ATF-2, a transcription factor downstream of p38 MAPK and JNK/SAPK was not consistently increased in the tumor tissue (data not shown) further suggesting that IGF-II does not utilize the JNK/SAPK pathway to induce tumorigenesis and that specific transcription factors downstream of p38 MAPK are activated by IGF-II in lung tumors. These results suggest that although different upstream signaling pathways may be utilized in individual tumors, convergence of these pathways on particular transcription factors is a more important event.

To determine the distribution of phosphorylated Erk1/Erk2, p38 MAPK and CREB within the lung tumors immunohistochemistry was performed. We found that lung tumor samples T2 and T3 that had high levels of phosphorylated Erk1/Erk2 by western analysis also had high levels of phosphorylated Erk1/Erk2 as detected immunohistochemically. The staining was observed in the nucleus of the tumor cells and was heterogenous with most of the staining appearing near the borders of the tumor (Fig. 5a). Lung tumors T1, T4 and T5 had no nuclear phospho-Erk1/Erk2 staining and only very faint cytoplasmic staining (Fig. 5b). Phosphorylated p38 MAPK could not be detected in any of the tumor tissue suggesting that the antibody was unable to detected phosphorylated p38 MAPK in our formalin-fixed tissue samples. Phosphorylated CREB was detected in all lung tumor tissue analyzed and displayed a heterogenous staining pattern with high levels of staining again near the outer edges of the tumors (Fig. 5c,d). Phosphorylated CREB was also found in tumors of the uterus (Fig. 5e), male accessory gland (Fig. 5f) and lacrimal gland (Fig. 5g) but not in the mammary tumors, luteomas or lymphomas. Therefore, activation of the transcription factor CREB appears to be a tissue-specific event in IGF-II-induced tumorigenesis.

In summary this study showed that overexpression of IGF-II was sufficient to induce sporadic tumors in a number of tissues and that sensitivity to IGF-II-induced tumorigenesis was not strictly dependent on IGF-II expression levels. Furhter, within the same organ, the lung, IGF-II-mediated tumorigenesis was associated with activation of different upstream signaling molecules. Although it remains possible that activation of Erk1/Erk2 or p38 MAPK in individual lung tumors represent independent signaling events, our data suggests that these different upstream signaling molecules converge and activate similar transcription factors. We found that activation of both the Erk1/Erk2 pathway and the p38 MAPK pathway in the IGF-II-induced lung tumors resulted in activation of the transcription factor CREB. This finding may have important therapeutic implications. Treatment modalities that target upstream signaling molecules may only be effective against a subset of tumors. In contrast, targeting downstream molecules

(i.e. transcription factors) may increase the chances of affecting a greater percentage of the tumors. In our study, treating the five lung tumors with an inhibitor of Erk1/Erk2 activity would likely be effective on only 2/5 (40%) of the tumors. However, if CREB activity could be effectively inhibited, 5/5 (100%) of the tumors would likely be responsive to the treatment. Thus, when genomic-based screening is employed to find common alterations within a class of tumors, the molecule furthest downstream in a signaling pathway may represent the most suitable therapeutic target.

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Figure Legends

Figure 1. Organ distribution of IGF-II expression and organ abnormalites. RNA was extracted from tissues of day 35-old female transgenic mice and reversed transcribed. The resulting cDNAs were amplified using a forward primer located in the MMTV promoter (5'-GCCATCCCGTCTCCGCTCGTCACTTATC-3') and reverse primer in the IGF-II transgene (5'-GCCTCCCTGAACGCCTCG-3') for 36 cycles. The samples were then separated on an agarose gel, transferred to nylon membrane and probed with ^{32}P-labeled H-*ras* probed as described[23]. (a) Transgene expression was detected in the mammary, kidney, uterus, ovary, liver, spleen, pancreas, and salivary. Wild type and transgenic mice at different ages were sacrificed and organs were fixed in 4%-buffered formalin. Following paraffin-embedding and sectioning the tissues were stained with hematoxylin and eosin and examined using a light microscope. The pie charts represent the distribution of histological alterations observed in (b) female and (c) male transgenic mice.

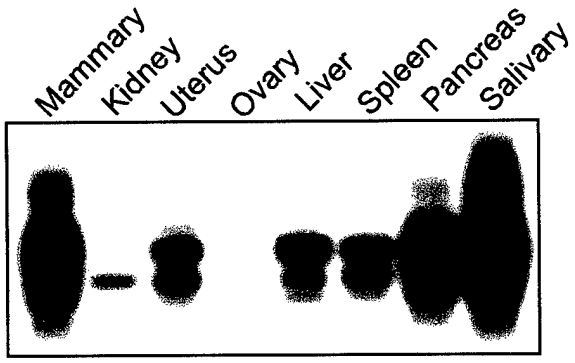
Figure 2. Histology of representative tumors. Hematoxylin and eosin stained sections of (A) lung tumor at 100x (B) lung tumor at 400x, (C) male accessory gland tumor at 100x, inset 400x, (D) uterine tumor at 100x, inset 630x, (E) Luteoma at 100x, inset 630x, (F) mammary tumor at 100x and (G) lymphoma of the lung at 100x. Immunohistochemical detection of (H) T cells and (I) B cells in a lung lymphoma using anti-CD3 and anti-B220 respectively. For immunohistochemistry formalin-fixed, paraffin-embedded sections were dewaxed in toluene and dehydrated in ethanol. Microwave antigen retrieval was performed by treating sections in citrate buffer at 95-100°C for 16 minutes. Sections were then incubated with anti-CD3 (Dako Diagnostics, Mississauga, ON, Canada) or anti-B220 (BD Pharmingen, San Deigo, CA) overnight at 4°C. The binding of primary antibodies was visualized using peroxidase-conjugated anti-rabbit or mouse IgG and counter-stained with haematoxylin.

Figure 3. Quantitative RT-PCR and in situ hybridization. (A) The levels of IGF-II expression were examined by extracting RNA from various organs from adult female transgenic mice. Following reverse transcription the resulting cDNAs were subjected to 23 cycles of PCR using primers specific for either transgenic IGF-II (5'-CCGAGAGGGACGTGTCGA-3' and 5'-GCCTCCCTGAACGCCTCG-3') or the house-keeping gene HPRT (5'-GTTGGATACAGGCCAGACTTTGTTG-3' and 5'-GATTCAACTTGCGCTCATCTTAGGC-3'). PCR products were transferred to nylon membrane and probed with ^{32}P-labeled IGF-II or HPRT probes. (B) Quantification of the levels of IGF-II relative to HPRT using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). In situ hybridization using a digoxigenen-labeled IGF-II riboprobe as described[23,33,34] {56,453,555}, was performed on embryonic day 16.5 (C) transgenic salivary, (D) transgenic lung, (E) wild type salivary, and (F) wild type lung as well as in tumors arising in (G) uterus, (H) lung, and (I) mammary. All images at 100x magnification and insets at 400x maginification.

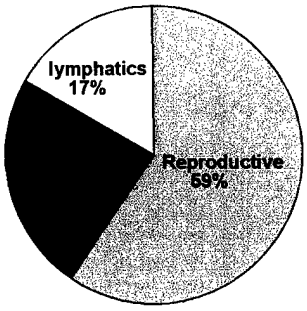
Figure 4. Westerns of Intracellular Signaling Molecules. Protein isolation and western blotting was performed as described[23]. Primary antibodies for Akt, phosphorylated Akt (Ser473), Erk1/Erk2, phosphorylated Erk1/Erk2 (Thr202/Tyr204), p38 MAPK, phosphorylated p38 MAPK (Thr180/Tyr182), ATF-2, phosphorylated ATF-2 (Thr71), CREB, phosphorylated CREB (Ser133) phosphorylated Elk-1 (Ser383), and phosphorylated JNK/SAPK (Thr183/Tyr185) (New England Biolabs,) were used at a dilution of 1:1000 in 5 % BSA in TBST. Membranes were incubated with the appropriate antibody overnight at 4°C. Proteins were detected using HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and LumiGLO reagents (New England Biolabs) and were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA). Sequential probing of membranes was performed after stripping in 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min at 50°C.

Figure 5. Immunohistochemical Detection of phosphorylated Erk1/Erk2 and CREB in Tumor Tissue. Formalin-fixed, paraffin-embedded sections were dewaxed in toluene and dehydrated in ethanol. Microwave antigen retrieval was performed by treating sections in citrate buffer at 95-100°C for 16 minutes. Sections were then incubated with anti-phospho-Erk1/Erk2 (Thr202/Tyr204, 1:200 dilution, New England Biolabs, Beverly, MA), anti-phospho-p38 MAPK (Thr180/Tyr182, 1:200 dilution, New England Biolabs) or anti-phospho-CREB (Ser133, 1:100 dilution, New England Biolabs) overnight at 4°C. The binding of primary antibodies was visualized using peroxidase-conjugated anti-rabbit or mouse IgG. All sections were counter-stained with haematoxylin.

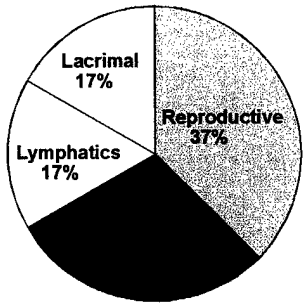
A

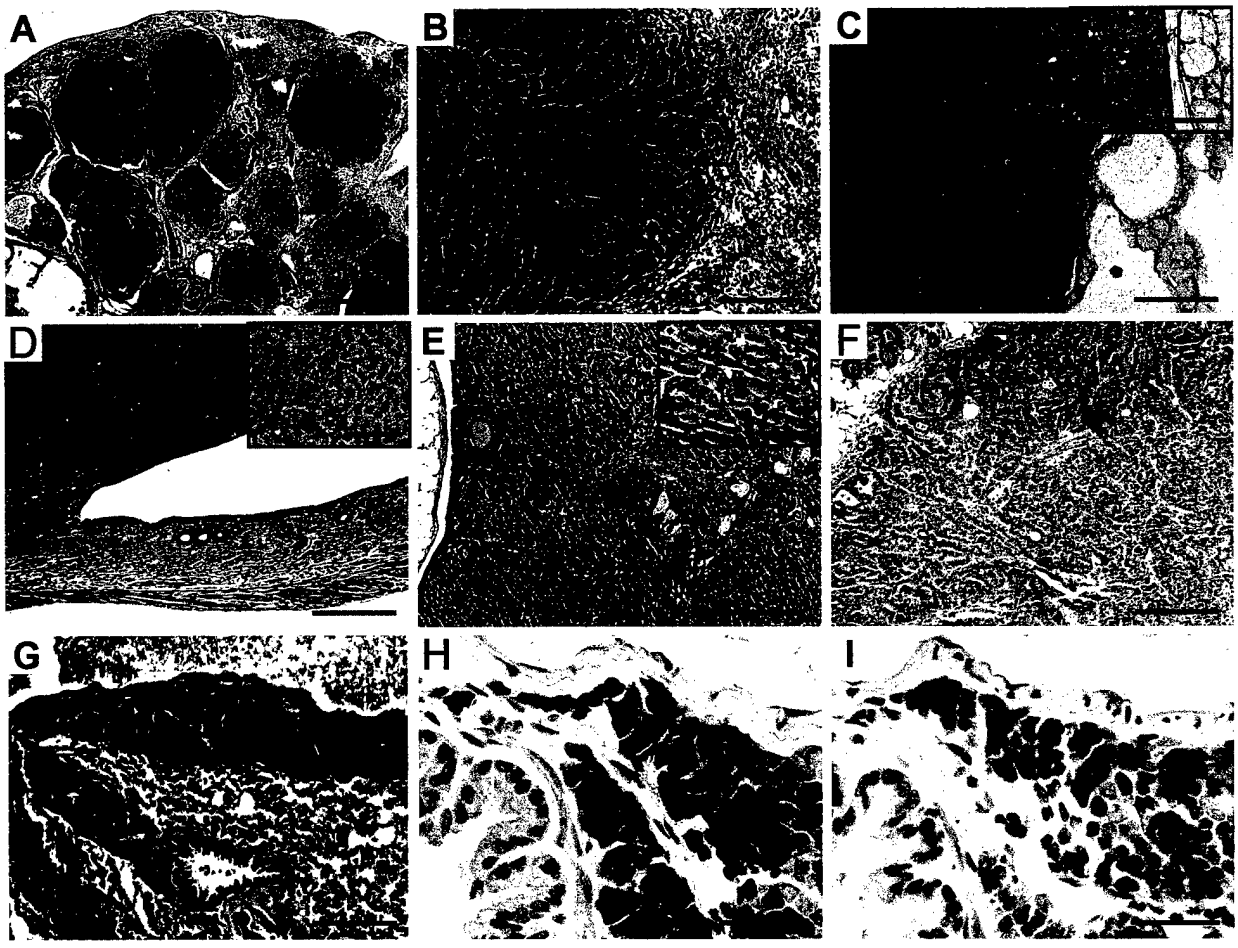


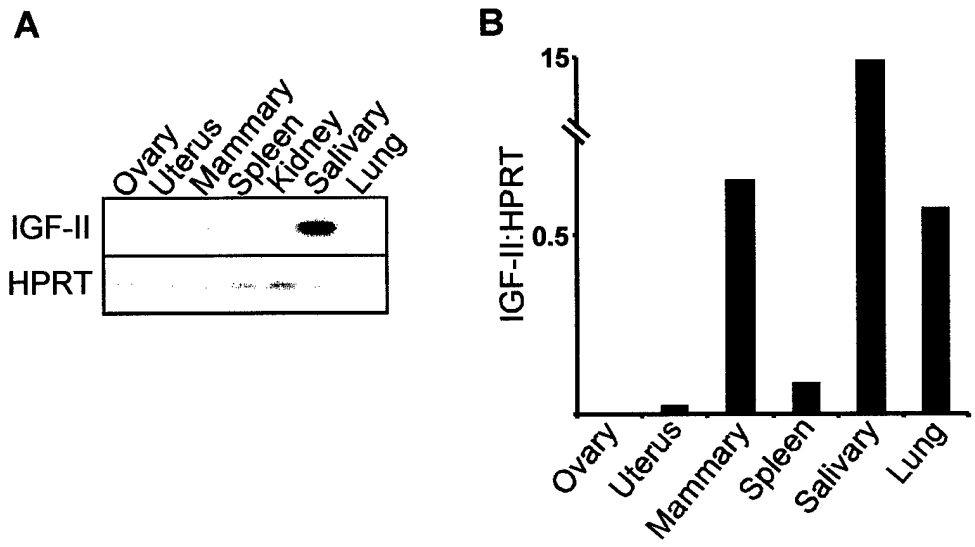
B Female

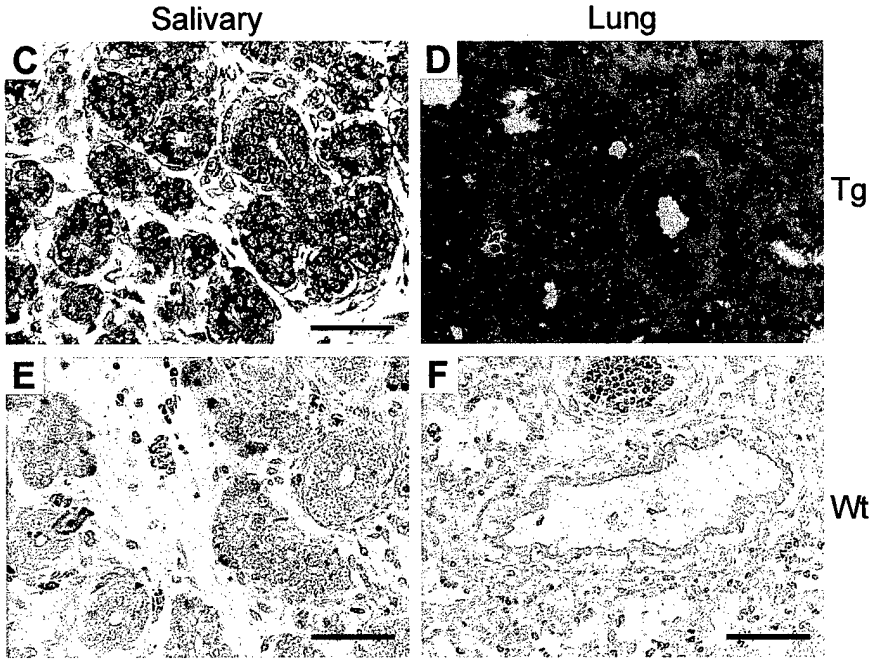


C Male




A
B
C
D
E
F
G
H
I


A
Ovary
Uterus
Mammary
Spleen
Kidney
Salivary
Lung
IGF-II
HPRT
B
15
0.5
IGF-II:HPRT
Ovary
Uterus
Mammary
Spleen
Salivary
Lung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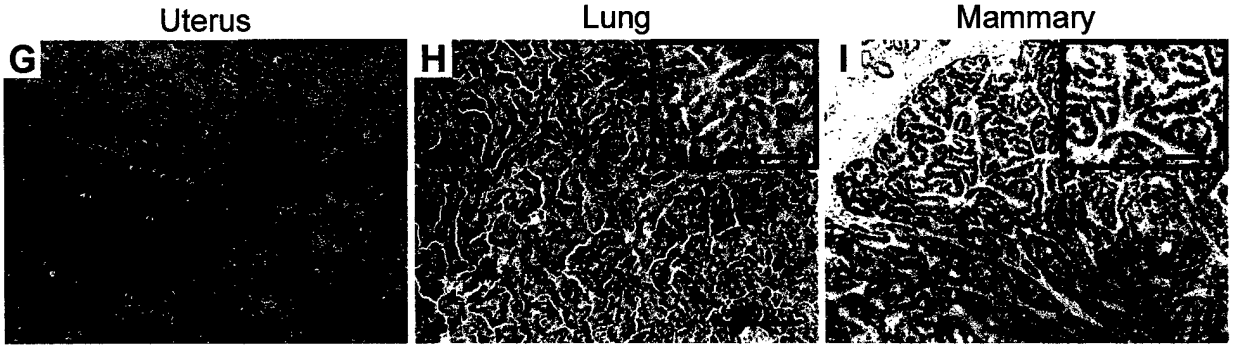
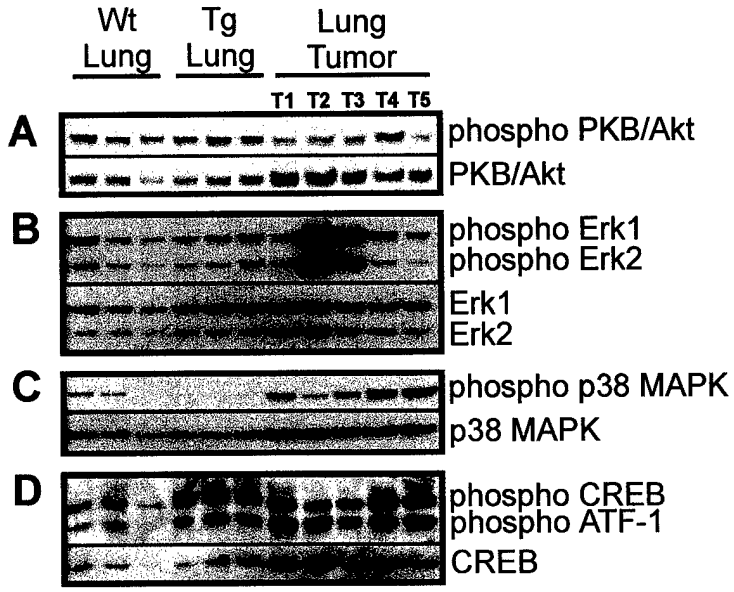
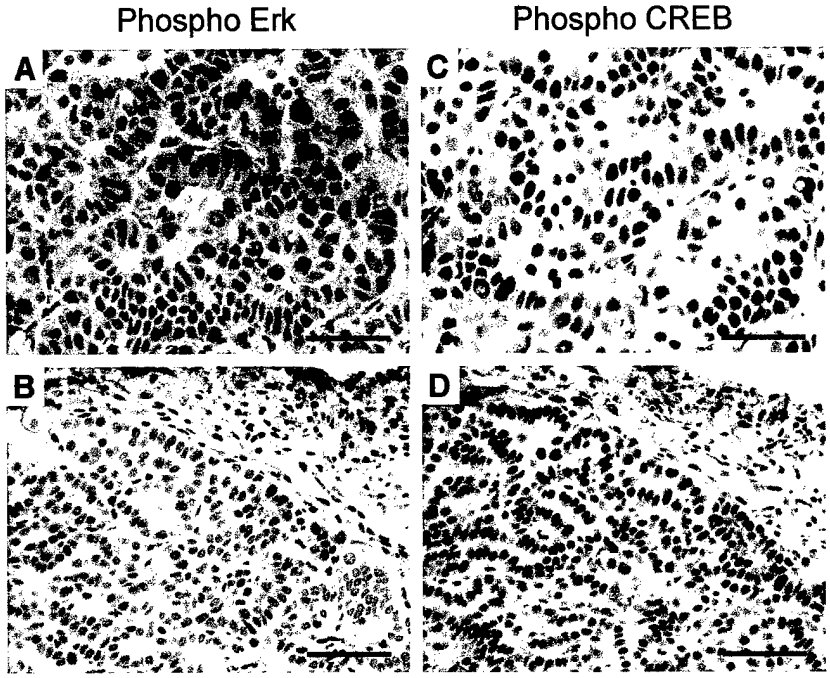
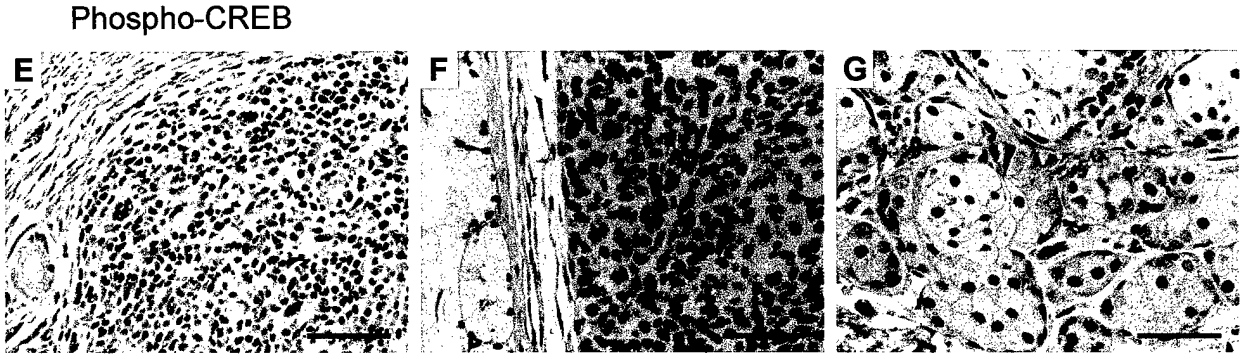
Lung
C
D
Tg
E
F
Wt


Uterus
Lung
Mammary
G
H
I


Wt Lung
Tg Lung
Lung Tumor
T1 T2 T3 T4 T5
A
phospho PKB/Akt
PKB/Akt
B
phospho Erk1
phospho Erk2
Erk1
Erk2
C
phospho p38 MAPK
p38 MAPK
D
phospho CREB
phospho ATF-1
CREB


Phospho Erk
Phospho CREB
A
C
B
D


Phospho-CREB
E
F
G